



Universidad
Zaragoza

Tesis Doctoral

Los cultivos protectores: estrategia *clean label*
en la industria alimentaria

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Programa de Doctorado en Calidad, Seguridad y Tecnología de los
Alimentos

Escuela de Doctorado

2025



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2025

Ayudas para la Contratación de Doctorandos y Doctorandas por Empresas, Centros de Investigación y Centros Tecnológicos "Doctorados Industriales 2022"

Gobierno de Navarra-Nafarroako Gobernua

Programa de Doctorado en Calidad, Seguridad y Tecnología de los Alimentos
Escuela de Doctorado UNIZAR



**Escuela
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**Departamento de
Producción Animal
y Ciencia de los Alimentos
Universidad Zaragoza**

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El viaje de la tesis ha estado lleno de emociones: tanto positivas como negativas, de risas como lloros, de seguridad e incertidumbre. Pero siempre ha estado lleno de personas que han hecho que este camino haya sido más fácil. Esta tesis no es solo un trabajo académico, sino también un reflejo de todo lo vivido, sentido y compartido durante estos años. Este proceso me ha permitido no solo crecer como investigadora sino también como persona. Además, me ha brindado la oportunidad de conocer a profesionales maravillosos en congresos y reuniones.

En primer lugar, quiero agradecer a mis directores de tesis, Raquel, Laura y Rafa por permitirme ser parte de este *tándem* que hemos formado. A Raquel, por ser una guía firme y exigente, pero también por preocuparte por mí más allá del trabajo. Gracias por darme el espacio y la confianza para proponer mis ideas, por escucharme y enseñarme. A Rafa, por ser una fuente constante de inspiración, con tu experiencia y trayectoria, siempre has encontrado un hueco para mí. Gracias por escucharme, orientarme y animarme. Gracias por tu generosidad, por compartir tu sabiduría con tanta cercanía y por hacerme sentir siempre bienvenida, sobre todo cuando estuve en Zaragoza. Y a Laura, la más joven de los codirectores, pero una auténtica inspiración para mí. Por cómo afrontas los retos, con valentía, inteligencia y una energía contagiosa. Aunque ha sido tu primera tesis, no me has soltado nunca, con una dedicación y una cercanía que han hecho que este camino haya sido más llevadero. Gracias por enseñarme tanto.

A mi *ama* y a mi *aita* que siempre han confiado en mí y ser el pilar más firme de mi vida. Gracias por haberme apoyado siempre, sin condiciones y por haber hecho todo lo posible para que tuviera la oportunidad que vosotros no pudisteis tener en cuanto a estudios. Una parte de esta tesis también es vuestra porque sin vuestro ejemplo de sacrificio, esfuerzo y amor incondicional, no estaría presentando esta tesis.

A mi otra mitad, mi hermana Iratxe: gracias por entenderme y chincharme a partes iguales, por hacerme reír y recordarme quién soy cuando he tenido bajones. Por tu apoyo, tu alegría y tu forma de ver la vida. *Mila esker, polita.*

A mi pareja Juan, con quién emprendí esta aventura al poco de conocerte, y que decidiste quedarte durante todos estos años. Gracias por acompañarme en este proceso tan complejo, por apoyarme en cada etapa, por celebrar mis logros y sostenerme en los momentos más difíciles. Gracias por tu paciencia, amor constante y por ayudarme a crecer. Tu presencia en mi vida es un equilibrio entre calma y motivación, entre tranquilidad e intensidad. Esta tesis es un ejemplo de lo que estamos construyendo juntos. *Mila esker, maitia.*

A mi cuadrilla, con quienes comparto recuerdos desde que teníamos tres años. Gracias por ser los primeros en animarme a embarcarme en esta locura, por creer en mí y por acompañarme con risas, consejos y cariño incondicional. Hemos vivido mil y una cosas juntos, pero aún nos queda mucho por compartir. *Mila esker beti nire ondoan egoteagatik, etxean bezala sentiarazteagatik eta nire bizitzako parte berezienetako bat izateagatik.*

Gracias de corazón a todos mis tíos y tías, tatas y tatos, y a mis *pitxones*, que cuando me junto con estos últimos, se me pasan todos los males al ver en sus miradas de amor y felicidad.

A mis compis de laboratorio, gracias por los cafés y las comidas compartidas, las conversaciones sobre ciencia que me hacían recapacitar, los viajes, las fiestas y el apoyo incondicional para llevar a cabo esta tesis, tanto dentro como fuera del laboratorio.

También quiero agradecer a las personas que conocí durante mi paso por la Universidad de Zaragoza; en especial a Natalia, Livia, Julia y Elisa. Gracias por acogerme con tanto cariño, por compartir vuestro tiempo, vuestras ideas y por hacer que esos meses fueran

tan especiales. Y a Esther, gracias por tu guía, tu apoyo y tu cercanía durante ese tiempo. Tu acompañamiento fue clave para que pudiera aprovechar al máximo esa experiencia.

Por último, quiero agradecer al CNTA, a UNIZAR y al Gobierno de Navarra por permitirme aportar mi granito de arena a la ciencia, gracias al apoyo financiero y material recibido.

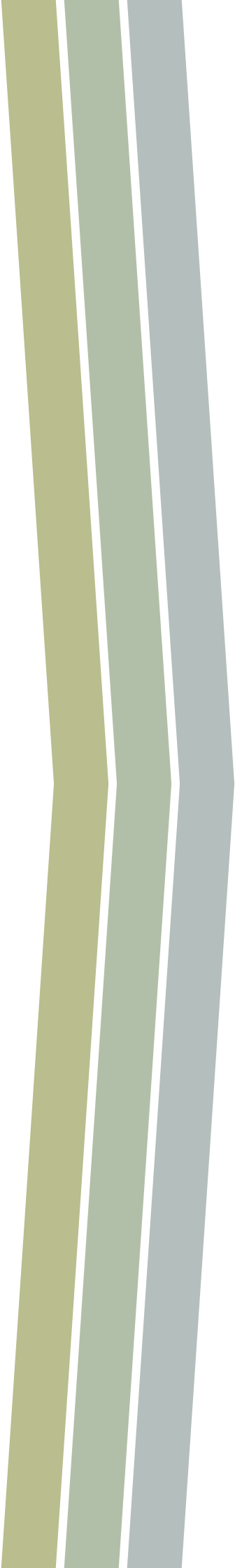
Gracias a todas y todos, sin vuestro apoyo nada de esto hubiera sido posible.

El desarrollo de esta tesis industrial ha sido posible gracias a la “Ayuda para la contratación de doctorandos y doctorandas, "Doctorados industriales 2022"” (convocatoria 2022) otorgada por el Gobierno de Navarra. Este proyecto también se benefició del apoyo y financiación del grupo de investigación de referencia A06-23-R-Análisis y Evaluación de la Seguridad Alimentaria (AESA), así como del Centro Nacional de Tecnología y Seguridad Alimentaria:

- **Proyecto BacBiop (0011-1408-2022-000003)** “Los cultivos protectores: estrategia *Clean Label* en la industria alimentaria”, Departamento de Universidad, Innovación y Transformación Digital, Servicio de I+D+i del Gobierno de Navarra (2022/2025), Investigador Principal: Nerea Garín Murguialday.
- **Proyecto PID2021-123404NB-I00** “Role of antibiotic-resistant, biocide-resistant, and preservation method-resistant *Salmonella* spp. variants in food safety”. Agencia Estatal de Investigación (AEI) (2022/2025), Investigador Principal: Diego García Gonzalo, Rafael Pagán Tomás.



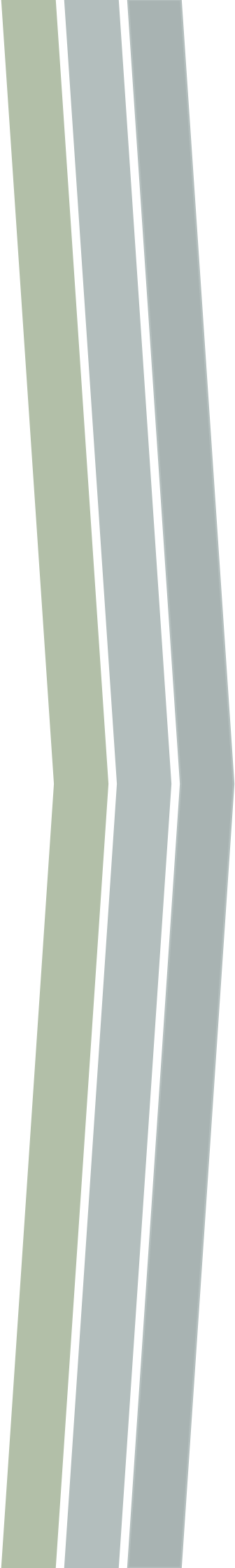
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RESUMEN



Resumen

Esta tesis doctoral, desarrollada bajo la modalidad de doctorado industrial, ha abordado de forma integral la selección, caracterización y validación de cultivos protectores y sus sobrenadantes libres de células (SLC) como estrategia *clean label* para la conservación microbiológica de alimentos mínimamente procesados. En estrecha colaboración con tres empresas alimentarias, se plantearon y resolvieron desafíos concretos vinculados al alargamiento de la vida útil de productos refrigerados, integrando herramientas de microbiología clásica, microbiología molecular y análisis bioinformático en un enfoque científico robusto y orientado a la transferencia tecnológica.

El trabajo experimental se dividió en tres etapas. En la primera, se cribaron más de 500 cepas de la colección del CNTA, aplicando criterios regulatorios (Protocolo de Nagoya, estatus QPS, resistencias a antibióticos y toxigenicidad) y funcionales (actividad antimicrobiana frente a microorganismos patógenos y alterantes relevantes para IV y V gama). Tras una caracterización genotípica y fenotípica rigurosa, se seleccionaron cuatro cepas: *Bacillus subtilis* CNTA 517, *Lactiplantibacillus plantarum* CNTA 600, *Pediococcus acidilactici* CNTA 1059 y *Levilactobacillus brevis* CNTA 1374. Sus SLC mostraron perfiles antimicrobianos diferenciados: las 3 bacterias ácido-lácticas produjeron altos niveles de ácido láctico (hasta 8,2 g/L), ácido acético (hasta 3,8 g/L) y, en el caso de *P. acidilactici*, también ácido feniláctico (0,119 g/L), compuesto con actividad antifúngica demostrada. Las cuatro cepas produjeron bacteriocinas de bajo peso molecular (<10 kDa), cuya presencia se confirmó mediante zimografía y espectrometría de masas.

La segunda etapa se centró en la caracterización tecnológica de los SLC, demostrando su actividad antimicrobiana frente a bacterias patógenas y alterantes incluso tras ser

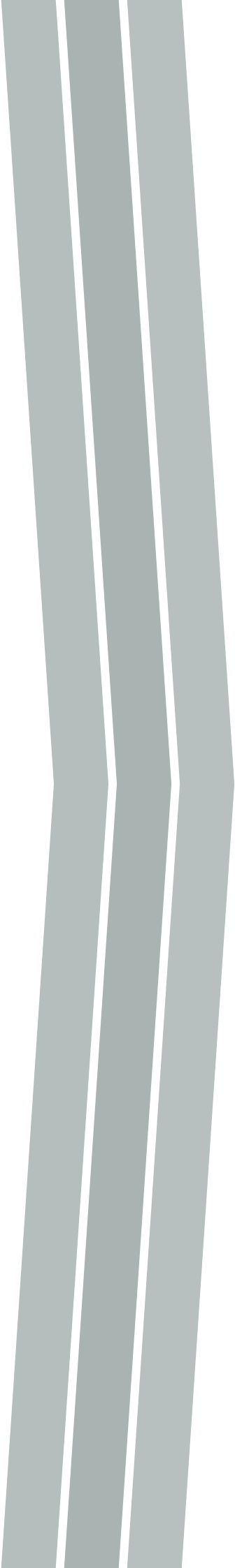
sometidos a tratamientos térmicos industriales habituales, incluida la cocción botulínica (120 °C/20 min), lo que amplía su aplicabilidad a productos pasteurizados y esterilizados. Esta estabilidad térmica confiere a los SLC un alto potencial como ingredientes de conservación natural.

En la tercera etapa se validaron los SLC en tres matrices reales proporcionadas por las empresas colaboradoras. En sticks de piña fresca el tratamiento por inmersión con el SLC de *P. acidilactici* CNTA 1059 (combinado con envasado semipermeable) permitió alargar la vida útil del producto de 8 a 12 días, con buena aceptación sensorial. En los bocados *plant-based* tipo “pollo”, el uso del SLC de *L. plantarum* CNTA 600 en formato líquido permitió extender la vida útil de 30 a 90 días, mostrando actividad frente a una bacteria del grupo *Bacillus cereus* y *Niallia circulans* aislados del producto alterado. En cambio, en las condiciones ensayadas, la aplicación del SLC de *L. brevis* CNTA 1374 en gazpacho no logró alcanzar el reto de duplicar la vida útil planteado, evidenciando la necesidad de optimizar su combinación con otras tecnologías de conservación.

Los resultados obtenidos demuestran no solo la eficacia práctica de los cultivos protectores seleccionados, sino también la solidez científica del proceso seguido, así como su aplicabilidad industrial en contextos reales. Esta tesis constituye una aportación relevante al conocimiento en bioconservación alimentaria, mostrando que el uso racional y científicamente fundamentado del SLC permite desarrollar soluciones tecnológicamente viables, sostenibles y alineadas con las demandas actuales del sector alimentario.



INTRODUCCIÓN



La introducción de esta tesis doctoral se ha estructurado y se presenta en forma de revisión científica, con el objetivo de ser sometida a evaluación para su posible publicación en una revista internacional de reconocido prestigio.

Manuscrito I

Bioprotective cultures as a clean label strategy for food preservation: from concept to market

Nerea Garín-Murguialday, Raquel Virto, Rafael Pagán and Laura Espina*

Title: Bioprotective cultures as a clean label strategy for food preservation: from concept to market

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Abstract

Bioprotective cultures represent a promising clean label strategy to enhance food safety and extend shelf-life while aligning with consumer demand for minimally processed products. This review examines the scientific principles, regulatory frameworks, mechanisms of action, and practical strategies for applying bioprotective cultures, with a special focus on lactic acid bacteria. Protective cultures act by inhibiting spoilage and pathogenic microorganisms through competitive exclusion, acidification, and the production of antimicrobial compounds such as bacteriocins, organic acids, and hydrogen peroxide. Various strategies for incorporating these cultures (direct addition into formulations, surface application through immersion or spraying, and their integration into edible coatings and active packaging) are explained throughout. Applications across diverse food matrices—including fruits, vegetables, dairy, cereals, meat, and seafood—demonstrate how bioprotective cultures can replace or complement conventional preservatives. As interest in clean label solutions continues to expand, the strategic use of bioprotective cultures is expected to play a key role in sustainable food preservation.

Keywords

Bioprotective cultures; food preservation; bacteriocins; biopreservation; clean label; lactic acid bacteria

1. Introduction

According to the 2022 Data & Trends of the EU Food and Drink Industry report, the food industry is the principal sector within the manufacturing industry, accounting for 14.3% of the total and being the foremost employer with 15.3%. This sector comprises 290,000 companies and employs 4.6 million people.

The main objective of this dynamic industry is to market safe and quality products. In this sense, each food product has its own microbiota, which may contain pathogenic and spoilage microorganisms. These microorganisms can be introduced at multiple stages of food production, whether through contaminated ingredients, inadequate hygiene practices, or cross-contamination during processing and storage (Alum et al., 2016). Foodborne pathogens (such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp.) are of particular concern due to their ability to cause severe foodborne illnesses, leading to significant public health risks and economic consequences (Ben et al., 2019). Spoilage microorganisms, on the other hand, cause negative organoleptic changes in food, resulting in financial impacts on producers, distributors, and consumers (Sevindik & Uysal, 2021). The presence of these spoilage microorganisms accounts for 30–40% of food waste, a challenge that both authorities and the industry aim to mitigate.

To mitigate the risks posed by the presence of pathogenic and spoilage microorganisms, the implementation of effective food preservation methods is essential. Methods that act by inhibiting microbial growth include refrigeration, freezing, drying, curing, vacuum packing, modified atmosphere packaging, acidification, fermentation, and the addition of preservatives (Gould, 2000). In contrast, thermal processes such as pasteurization and sterilization act by directly inactivating microorganisms. Additionally, aseptic processing and packaging further limit microbial access to food products (Gould,

2000). Due to consumer preferences shift towards minimally processed foods, emerging or modern technologies are being developed (Strack et al., 2020). These include preservation by high hydrostatic pressure (Aganovic et al., 2021), pulsed electric fields (García et al., 2003), ozone (Pandiselvam et al., 2019) and plasma (López et al., 2019). Essential oils and their individual constituents, known for their antimicrobial properties, are also being explored as natural preservatives in food systems due to their ability to inhibit bacterial and fungal growth (Corrêa & Ferreira, 2023). Other nature-extracted preservatives, such as propolis (Luis-Villaroya et al., 2015) or chitosan (Raafat & Sahl, 2009), are also gaining interest due to their broad-spectrum antimicrobial activity and potential for application in different food matrices. These preservatives can be incorporated directly into food, encapsulated in nanoemulsions for controlled release (Maurya et al., 2021; Pagán et al., 2018), or integrated into edible coatings to enhance preservation (W. Zhang et al., 2022). Combined preservation techniques (also referred to as hurdle technology) are widely recognized for their effectiveness in inhibiting microbial growth and survival without significantly compromising the organoleptic and nutritional qualities of food (Leistner, 2000). This strategic simultaneous or sequential application of multiple preservation methods, like essential oils and heat or other physical hurdles, has been successfully applied in various food products such as fruits, vegetables and juices, fish and fish products, and meat and meat products (Aaliya et al., 2021).

Either used alone or as part of combined preservation strategies, chemical additives are often perceived by consumers as incompatible with the demand for “natural” and “healthy” food (Negowetti et al., 2022). Current consumer preferences reflect a growing emphasis on health, sustainability, and transparency, driving demand for *clean-label* foods, which are mainly understood as minimally processed and without additives, preservatives, artificial colors, or ingredients (Aschemann-Witzel et al., 2019; Cegiela,

2020; Negowetti et al., 2022). Simultaneously, the popularity of convenient, ready-to-eat foods continues to increase (RTE) (Aschemann-Witzel et al., 2019; Asioli et al., 2017). In this context, biopreservation offers a promising alternative to conventional preservatives that aligns with these expectations by enabling the development of *clean-label* foods that are both convenient and have a long shelf life (Anumudu et al., 2022).

This manuscript provides an overview of the use and application methods of protective cultures as an alternative to conventional preservatives proposed by the food industry in order to satisfy the needs of both consumers and the industry, addressing the aforementioned reasons, and ensuring the safety, quality, and extended shelf-life of products within the context of the clean label trend.

2. Protective cultures: science and regulations

2.1. Concept of protective cultures

Biological preservation refers to the extension of the shelf-life of food products and improvement of their microbial safety by the use of natural or controlled microbiota and/or their antimicrobial compounds (Ross et al., 2002). When specific strains of microorganisms (typically bacteria or yeasts) are intentionally added to a food product to inhibit the growth of spoilage and pathogenic microbes, they are referred to as protective cultures (Leroy & De Vuyst, 2004). The preservative effect works by inhibiting the growth of undesirable microorganisms (such as foodborne pathogens and spoilage bacteria) through natural mechanisms like competitive exclusion, acidification, and antimicrobial compound production (e.g., bacteriocins, organic acids, or hydrogen peroxide) (Ayala et al., 2019; Gálvez et al., 2007; Leroy & De Vuyst, 2004).

Although both protective and starter cultures involve the intentional addition of selected microorganisms, their purposes differ. Starter cultures are used to drive

fermentation processes that actively transform the food's sensory characteristics—such as texture, flavor, and pH—through microbial metabolism. In contrast, protective cultures are primarily aimed at preventing microbial spoilage and contamination without significantly altering the product's sensory properties. Their function is centered on preservation and food safety rather than fermentation-driven changes.

2.2. Regulatory frameworks for the production of protective cultures

The commercialization of bioprotective cultures in food production is tightly regulated to ensure microbial safety, functional efficacy, and compliance with regional food laws. Regulatory frameworks differ significantly across different countries, reflecting distinct approaches to risk assessment, approval processes, and labeling requirements. This review is centered on the regulatory frameworks in the European Union, but more information on the legal regulation in other areas can be found in a review by (Fischer & Titgemeyer, 2023).

In the European Union, the European Food Safety Authority (EFSA) conducts independent scientific evaluations to assess the safety of microorganisms used in food, including protective cultures. Based on EFSA's risk assessments, the European Commission establishes legally binding regulations governing their use in the food industry. The Commission, in collaboration with EU member states, is responsible for final regulatory approvals and enforcement under specific regulations such as the Novel Food Regulation (EU) 2015/2283 and the food additive legislation (EC) No 1333/2008.

2.2.1. The Qualified Presumption of Safety (QPS) System

The Qualified Presumption of Safety (QPS) is a pre-market safety assessment system established by the EFSA in 2007 to facilitate the approval of microorganisms intentionally introduced into the food and feed chain, harmonizing risk assessments across EFSA's Scientific Panels (EFSA, 2007; Herman et al., 2019).

This process assesses microorganisms based on four key aspects: taxonomic identity, the body of relevant knowledge, potential safety concerns, and the end use. (EFSA, 2007; Herman et al., 2019). For a TU to be considered for QPS status, it must be unambiguously identified using robust genomic methods, such as whole-genome sequencing (EFSA, 2024). For bacteria and yeasts, the lowest TU for which QPS status is granted is the species level (EFSA, 2007). After this taxonomic identification, the body of knowledge regarding the history of use, industrial applications, interaction with other microorganisms, etc. (Herman et al., 2019) is investigated based on the scientific literature. The assessment also evaluates the possible presence of virulence factors that could cause pathogenicity in humans or animals, as well as the production of biologically active substances like antimicrobials and toxins. Finally, it should be noted that the QPS assessment primarily focuses on the deliberate introduction of viable microorganisms. If a TU is used only for the production of specific compounds and no viable cells are present in the final product, it may receive a "for production purpose only" qualification. The list of QPS microorganisms is maintained and re-evaluated approximately every 6 months in a Biohazard Panel Statement (BIOHAZ et al., 2025).

2.2.2. Strain-level assessment

Even after a TU is granted QPS status, a strain-level assessment is required for individual strains. This includes confirming the unambiguous identification of the strain and verifying that it fulfills any qualifications. For bacterial strains, this primarily involves confirming the absence of acquired antimicrobial resistance genes to clinically relevant antimicrobials (EFSA BIOHAZ et al., 2023). In contrast, intrinsic antimicrobial resistance is not considered a risk factor. To help distinguish these, EFSA's 2023 guidelines now mandate whole-genome sequencing of at least 30 strains (EFSA BIOHAZ et al., 2023). This genomic analysis is complemented by phenotypic confirmation through

minimum inhibitory concentration (MIC) testing against clinical breakpoints. Additionally, the absence of toxigenic activity is a qualification for *Bacillus* species, meaning individual strains must be evaluated for the production of toxic compounds (EFSA FEEDAP, 2014).

2.2.3. Impact of QPS status on the regulatory pathway

QPS status significantly influences the regulatory pathways for microorganisms used in food and feed. QPS-listed strains benefit from an expedited approval process, as their QPS status eliminates the need for a full safety assessment at the TU level (Herman et al., 2019). This considerably shortens the approval timeline compared to non-QPS strains or novel foods. In contrast, non-QPS strains fall under the Novel Food Regulation (EU 2015/2283), necessitating a comprehensive dossier with detailed toxicology, exposure, and antimicrobial resistance data.

2.2.4. Criteria to be considered a protective culture in Europe

Within the European Union's regulatory framework (European & Council of the European, 2008), microbial cultures intended for use as protective cultures in the food industry must comply with a series of scientific and regulatory criteria to ensure its safety, efficacy, and technological applicability (Figure 1). These criteria are outlined in EFSA's guidance which includes, among others, the requirement for inclusion in the Qualified Presumption of Safety (QPS) list maintained by the European Food Safety Authority (EFSA) (EFSA BIOHAZ Panel, 2024). This designation implies a well-established taxonomic identity, a history of safe use, and the absence of safety concerns. Protective cultures must demonstrate antimicrobial activity against foodborne pathogens and/or spoilage organisms, while not producing toxins or harbouring virulence-associated genes, particularly in genera such as *Bacillus* and *Enterococcus* which require additional safety evaluations. Furthermore, strains must be susceptible to clinically relevant antibiotics and

free from transferable antibiotic resistance genes. Their application must not adversely impact on sensory properties of the final product and must be technologically suitable, remaining stable and effective throughout food processing and storage (Ben Said et al., 2019).

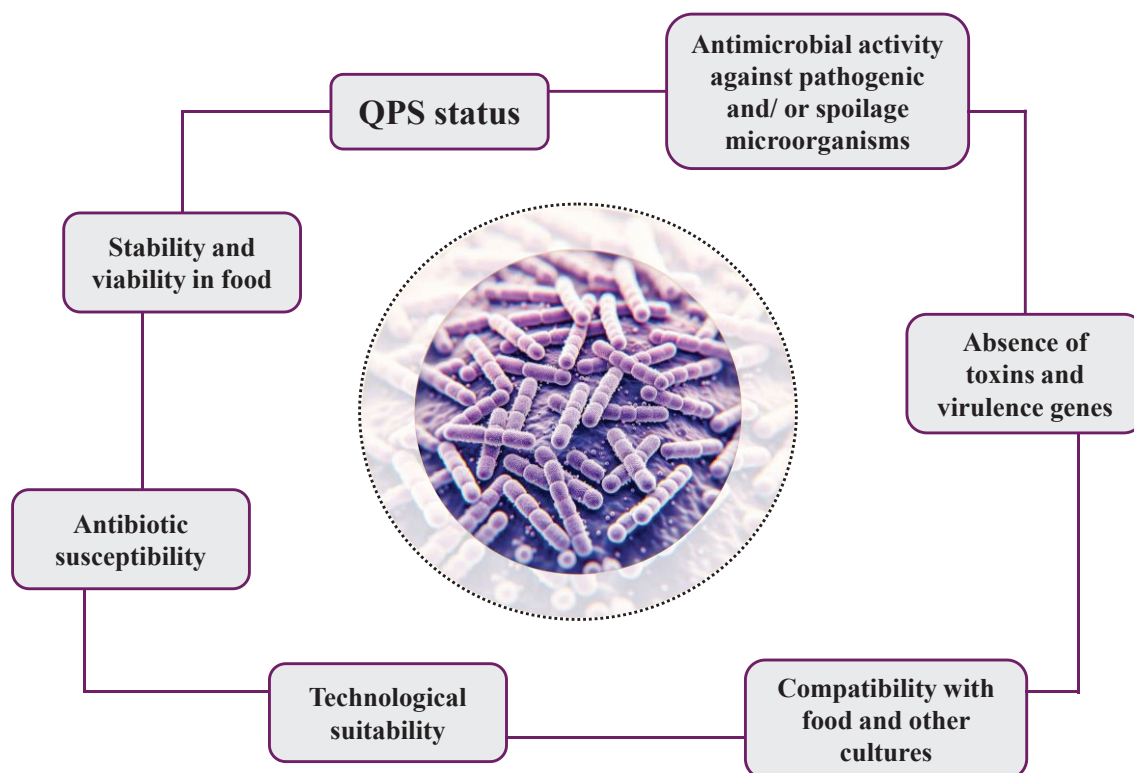


Figure 1. European Commissions requirements for microorganisms to be protective cultures

2.2.4. The Nagoya Protocol

The Nagoya Protocol (European & Council of the European, 2014) establishes a framework for Access and Benefit-Sharing of genetic resources, which is of relevance when considering the origin of the cultured microbial species. It mandates that genetic resources, including those utilized in protective cultures for the food industry, are accessed legally from the providing country with "Prior Informed Consent", and that "Mutually Agreed Terms" for benefit-sharing are in place. This aims to prevent "biopiracy" and ensure that benefits arising from the utilization of these genetic resources are shared fairly with the countries and often local communities that provided them,

fostering biodiversity conservation and sustainable use. For the EU food industry, this translates into rigorous documentation, traceability, and contractual obligations when sourcing or developing protective cultures derived from genetic resources.

2.3. Key Protective Cultures in the Food Industry

Among the diverse array of microorganisms employed for biopreservation, lactic acid bacteria (LAB) stand out as the most known and widely utilized (Ayivi et al., 2020; de Souza et al., 2023).

2.3.1. Lactic Acid Bacteria (LAB)

These are Gram-positive, non-spore-forming, facultative anaerobic or aerobic bacteria capable of fermenting the free sugars of the medium or food in which they are found, producing lactic acid as the major end product.

2.3.1.1. Classification and taxonomy of LAB

Depending on their carbohydrate fermentation pathways, LAB can be classified into homofermentative and heterofermentative (Mokoena, 2017). Homofermentative LAB, such as *Lactococcus*, *Streptococcus* and *Pediococcus*, primarily yield two molecules of lactate from one glucose molecule. In contrast, heterofermentative LAB, including *Leuconostoc*, *Weissella*, and some *Lactobacillus* species, produce lactate, ethanol, and carbon dioxide from a single glucose molecule (Ayivi et al., 2020; Mokoena, 2017).

It should be noted that the genus *Lactobacillus*, which is particularly relevant in biopreservation due to its diverse antimicrobial capabilities and its wide distribution across various fermented foods (Ayivi et al., 2020), has undergone a significant taxonomic reclassification (Zheng et al., 2020), summarized in Table 1.

Table 1. New names of *Lactobacillus* spp. Adapted from Ayivi et al., 2020.

Old name	New name
<i>Lactobacillus jinshani</i>	<i>Acetylactobacillus jinshani</i>
<i>Lactobacillus fermenti</i>	<i>Agrilactobacillus fermenti</i>
<i>Lactobacillus amylophilus</i>	<i>Amylolactobacillus amylophilus</i>
<i>Lactobacillus apinorum</i>	<i>Apilactobacillus apinorum</i>
<i>Lactobacillus bombi</i>	<i>Bombilactobacillus bombi</i>
<i>Lactobacillus alimentarius</i>	<i>Companilactobacillus alimentarius</i>
<i>Lactobacillus carnosus</i>	<i>Dellaglioia carnosus</i>
<i>Lactobacillus catenaformis</i>	<i>Eggerthia catenaformis</i>
<i>Lactobacillus fluorum</i>	<i>Fructilactobacillus fluorum</i>
<i>Lactobacillus fructosus</i>	<i>Fructobacillus fructosus</i>
<i>Lactobacillus rossiae</i>	<i>Furfurilactobacillus rossiae</i>
<i>Lactobacillus floricola</i>	<i>Holzappelia floricola</i>
<i>Lactobacillus vitulinus</i>	<i>Kandleria vitulinus</i>
<i>Lactobacillus casei</i>	<i>Lactocaseibacillus casei</i>
<i>Lactobacillus pentosus</i>	<i>Lactiplantibacillus pentosus</i>
<i>Lactobacillus johnsonii</i>	<i>Lactobacillus johnsonii</i>
<i>Lactobacillus xylosus</i>	<i>Lactococcus lactis</i>
<i>Lactobacillus concavus</i>	<i>Lapidilactobacillus concavus</i>
<i>Lactobacillus curvatus</i>	<i>Latilactobacillus curvatus</i>
<i>Lactobacillus parabuchneri</i>	<i>Lentilactobacillus parabuchneri</i>
<i>Lactobacillus brevis</i>	<i>Levilactobacillus brevis</i>
<i>Lactobacillus salivarius</i>	<i>Ligilactobacillus salivarius</i>
<i>Lactobacillus fermentum</i>	<i>Limosilactobacillus fermentum</i>
<i>Lactobacillus nagelii</i>	<i>Liquorilactobacillus nagelii</i>
<i>Lactobacillus coryniformis</i>	<i>Loigolactobacillus coryniformis</i>
<i>Lactobacillus vaccinoferus</i>	<i>Paucilactobacillus vaccinoferus</i>
<i>Lactobacillus perolens</i>	<i>Schleiferilactobacillus perolens</i>
<i>Lactobacillus oryzae</i>	<i>Secundilactobacillus oryzae</i>
<i>Lactobacillus confusus</i>	<i>Weissella confusus</i>

2.3.1.2. Mechanism of action of antimicrobial compounds of BAL

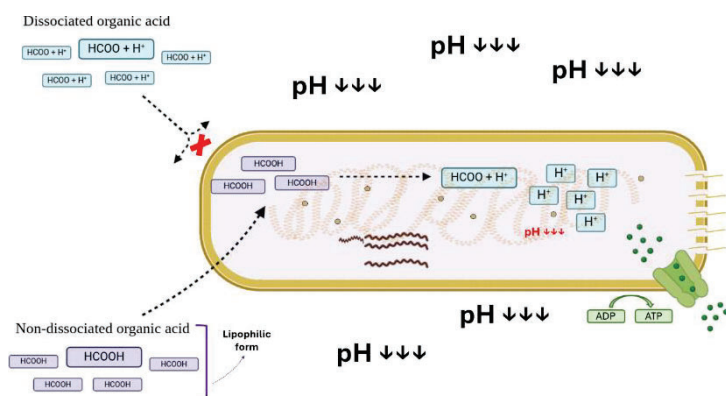
LAB, through different metabolic processes, can produce various compounds with broad-spectrum antimicrobial activity such as organic acids, hydrogen peroxide, bacteriocins, diacetyl, and ethanol (Ayivi et al., 2020; Ben Said et al., 2019; de Souza et al., 2023). Given that the first three are considered the primary and most extensively studied antimicrobial compounds contributing to LAB's biopreservative effects, a detailed explanation of their mechanisms of action is offered below.

2.3.1.2.1. Organic acids

LAB can produce a wide range of organic acids, such as lactic acid, acetic acid, and propionic acid, among others. These acids are obtained after fermentation of the carbohydrates present in the matrix (Ben Said et al., 2019; de Souza et al., 2023). The concentration of these acids depends on the bacterial strain, as well as the pH and temperature of the medium, and their interactions (Bangar et al., 2022).

The antimicrobial activity of organic acids is attributed to several mechanisms, including the disruption of nutrient absorption and the reduction of adenosine triphosphate (ATP) production (Kovanda et al., 2019). However, the most widely accepted explanation is the weak organic acid theory. According to this theory, at low pH, the undissociated (lipophilic) forms of organic acids can readily penetrate the cell membrane of pathogenic and spoilage bacteria. Once inside the cell (where the pH is typically higher), these acids dissociate, releasing H^+ ions, which lowers the intracellular pH, leading to cellular damage and forcing the cell to expend significant ATP to pump out the excess protons and maintain pH homeostasis (Bangar et al., 2022; Ben Braïek & Smaoui, 2021; Kovanda et al., 2019). The accumulated anions within the cell can also be toxic, inhibiting essential metabolic reactions, increasing osmotic pressure, and damaging cytosolic enzymes (Ben Braïek & Smaoui, 2021; Gómez-García et al., 2019; Yoon et al., 2024).

Figure 2. Intracellular mechanism of action of the organic acid through membrane diffusion and proton release (Figure created by the authors based on conceptual models from previously published literature).



The antimicrobial efficacy of organic acids is highly dependent on their chemical characteristics, particularly their pKa values, and the concentration of their undissociated forms. The pH of the surrounding medium significantly influences the ratio of dissociated to undissociated acid forms, especially near the acid's pKa, thereby dictating their inhibitory effectiveness. Generally, toxicity is strongly correlated with an increase in the concentration of protonated free acids and the pKa of the specific acid utilized (Braïek & Samoui, 2021; Gómez-García et al., 2019; Yoon et al., 2024).

Organic acids exhibit antimicrobial properties that are contingent upon their chemical characteristics, including their pKa values, as well as the concentration of their undissociated forms. At low pH, undissociated acid molecules, which are lipophilic, can cross microbial cell membranes and once inside the cell, these acids dissociate, releasing H⁺ ions that lower the intracellular pH, leading to cellular damage. The resultant anions are toxic, inhibiting metabolic reactions, increasing osmotic pressure, and damaging cytosolic enzymes. The pH of the medium influences in the determination of the ratio between the dissociated and undissociated forms of an acid near its dissociation constant (pKa), thereby influencing the inhibitory efficacy of organic acids. The toxicity of these acids is strongly correlated with an increase in the concentration of protonated free acids and the pKa of the acids utilized (Ben Braïek & Smaoui, 2021; Gómez-García et al., 2019; Yoon et al., 2024).

2.3.1.2.2. Hydrogen peroxide

In the presence of oxygen, LAB produce hydrogen peroxide due to the action of flavoprotein oxidase and/or nicotinamide adenine dinucleotide hydrogen peroxide (NADH) peroxidases. This can lead to the formation of free radicals and hydroxyl radicals, which are precursors to oxidative damage. Such damage permeabilizes the membrane and damages the DNA of target bacteria (Achi et al., 2019; Ben Said et al.,

2019). Optimal production of hydrogen peroxide by LAB occurs at 37°C and pH 5.5 (Ben Said et al., 2019).

2.3.1.2.3. Bacteriocins

While bacteriocins are produced by a wide array of bacteria, including notable examples from Gram-negative species, this section will primarily focus on the bacteriocins synthesized by LAB. Bacteriocins are a diverse group of antimicrobial peptides and proteins ribosomally synthesized by various bacterial species, many of them belonging to the BAL group. These non-toxic compounds are primarily known for their inhibitory activity against other often closely related bacterial strains, playing a crucial role in microbial competition within diverse environments (Cintas et al., 2001; da Costa et al., 2019).

While many bacteriocins exhibit a narrow spectrum, targeting specific species or genera, others, like nisin, demonstrate broader efficacy against a range of Gram-positive pathogens and spoilage microorganisms (Cotter et al., 2013; da Costa et al., 2019). A key characteristic of bacteriocins is their sophisticated self-protection mechanisms, ensuring that the producing bacteria remain unaffected by their own antimicrobial agents (da Costa et al., 2019; Lahiri et al., 2022). Furthermore, many bacteriocins, particularly those from food-grade bacteria, are generally considered safe for human consumption. They are often susceptible to inactivation by digestive enzymes, minimizing potential adverse effects on the human gut microbiota (Nes et al., 2007). Consequently, bacteriocins are garnering significant interest as promising antimicrobial alternatives in various fields, offering solutions to challenges such as antibiotic resistance and food spoilage (Cotter et al., 2013; Simons et al., 2020; Sugrue et al., 2024).

The production of bacteriocins is a complex biological process. While some are thermoresistant, their synthesis is often induced by environmental stress conditions, such

as high cell density (population increase) or nutrient limitation (Cintas et al., 2001). Their production is also significantly influenced by the availability of carbon and nitrogen sources in the growth medium and is intricately regulated by quorum sensing, a cell-to-cell communication system (da Costa et al., 2019; Todorov et al., 2022). Besides, they are generally produced during or at the end of the exponential growth phase, with their synthesis often linked to biomass production and regulated by inducing peptides or pheromones (da Costa et al., 2019; Todorov et al., 2022).

The genetic information encoding bacteriocins, along with their associated processing and immunity proteins, is typically organized into operon clusters. These gene clusters can be located on the bacterial chromosome, plasmids, or mobile genetic elements, highlighting their adaptability and horizontal transfer potential. Their expression is inducible, often requiring specific regulatory signals for both secretion and extracellular accumulation of the active peptides (Chanos & Mygind, 2016). While the primary antimicrobial mechanisms often involve pore formation in bacterial membranes or degradation of the cell wall, the exact modes of action can vary widely depending on the bacteriocin class. For instance, the mechanisms by which certain bacteriocins exert their effects against Gram-negative bacteria, which possess an outer membrane as an additional barrier, are more complex and require further comprehensive investigation (Pérez-Ramos et al., 2021).

Bacteriocins are currently grouped into several major classes based on their molecular size, stability, and mechanisms of action. This classification provides a useful framework for understanding their potential applications in food preservation and safety. Table 2, synthesized from recent literature (Benítez-Chao et al., 2021; Singh, 2018), summarizes the main bacteriocin classes and their description, structural features, mode of action and representative examples.

Table 2. Updated classification of bacteriocins based on structural features, mode of action, and representative examples.

Class	Description	Structural Features	Mode of Action	Bacteriocin examples
Class I	Lantibiotics	Modified amino acids	Pore formation	Nisin
Class IIa	Non-lantibiotic peptides. Antilisterial, Pediocin-like	Small, heat-stable, linear with bisulfide bridges	Pore formation	Pediocin PA-1
Class IIb	Non-lantibiotic peptides. Two-peptide	Two peptides	Pore formation	Lacticin 3147
Class IIc	Non-lantibiotic peptides. Circular	Circular peptides, with one or two cysteine residues	Pore formation	Enterocin AS-48
Class IId	Non-lantibiotic peptides. Linear non-pediocin	Linear peptides	Pore formation	Bacteriocin 31
Class III	Large, heat-labile	Large proteins	Enzymatic activity	Helveticin J
Class IV	Complex with lipids/carbohydrates	Complex structures	Varied	Leucocin S

Although information about bacteriocins is increasingly available, the most challenging aspect lies not in the technical aspect but in the legal one (Barcenilla et al., 2022). This challenge stems from the complexity of approving new bacteriocins as food additives and the necessity to determine the precise concentration of each bacteriocin to demonstrate antimicrobial activity in various foods without causing undesirable changes in the final product, such as alterations in color or flavor (Achi et al., 2019). For their use to be approved, extensive research on bacteriocin immunogenicity and toxicity is essential to confirm their safety. Additionally, clinical studies are required for the approval of bacteriocins by the World Health Organization and regulatory agencies (Parada Fabián et al., 2025).

Currently, the only food-grade bacteriocin authorized by EFSA, such as E-234, and by the FDA is nisin, which was approved in 1988. It exhibits antimicrobial activity against bacterial spores and Gram-positive bacteria, including *Clostridium botulinum*, *Listeria monocytogenes*, and *Staphylococcus aureus*. It is marketed as a partially purified product for use in both dairy and non-dairy fermentations (Soltani et al., 2021).

In addition to lactic acid bacteria, there are now other microorganisms described as protective cultures. Among them are the following species.

2.3.2. *Bacillus subtilis*

Bacillus subtilis, a Gram-positive, endospore-forming bacteria, has emerged as a protective culture due to its well-recognized potential as a biocontrol agent, contributing to shelf-life extension by exerting antimicrobial activity against pathogenic and/or spoilage microorganisms (Hinarejos et al., 2016; Rocha et al., 2023). Its application is primarily focused on crop and plant biocontrol (Ben Khedher et al., 2020), although its use has also expanded to minimally processed products, fruits and vegetables.

The primary mechanism of action of this bacterium is based on the production of lipopeptides secondary metabolites with antimicrobial activity, such as surfactin, iturin, fengycin, bacillomycin, and bacilysin, among others (Rocha et al., 2023; Jiawen Xiao et al., 2021). These compounds exhibit bactericidal and fungicidal activity by disrupting the cellular membrane integrity of bacterial pathogens such as *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis or *Staphylococcus aureus* (T. Chen et al., 2023; Fernandes et al., 2007). They have also been described as active against moulds like *Fusarium oxysporum* (Assena et al., 2024) or *Botrytis cinerea* (Jiling Xiao et al., 2023). Additionally, *B. subtilis* is capable of forming biofilms on food-contact surfaces, enabling it to compete for nutrients with other microorganisms and thereby inhibit their growth (Bridier et al., 2011; Carrascosa et al., 2021).

2.3.3. Others

Not only bacteria are used as protective cultures, but certain yeasts and moulds also have shown a significant role as natural preservatives in clean label products. Although they do not produce classical bacteriocins like LAB or *B. subtilis*, they do synthesize other

antimicrobial compounds that contribute to the inhibition of pathogenic and spoilage microorganisms in food.

One of the most recognized yeasts is *Debaryomyces hansenii*, a halotolerant species naturally present in cured meats and cheeses. Its protective role is based on competition for nutrients and space, as well as the production of antimicrobial compounds such as volatile fatty acids, ethanol, and phenolic compounds, which modify the surface microenvironment (Ramos-Moreno et al., 2021; L. Zhao et al., 2022). Another well-known yeast is *Saccharomyces cerevisiae*, widely used in the fermentation of products such as beer and bread (El Dana et al., 2025). It can also exert a protective effect through the production of compounds like ethanol, carbon dioxide, and proteinaceous toxins that inhibit the growth of other microorganisms (El Dana et al., 2025; Fakruddin et al., 2017).

Certain moulds, such as *Penicillium nalgiovense* and *Penicillium chrysogenum*, are used in meat and dairy products. These moulds exhibit both a competitive exclusion effect (their rapid growth on the food surface quickly depletes available nutrients and oxygen) and synthesis of specific antifungal and antibacterial metabolites (Guzmán-Chávez et al., 2018; Papagianni & Papamichael, 2007).

2.4. Strategies for applying bioprotective cultures in food

The increased use of protective cultures as food preservation ingredients is evidenced by the growing innovation within the industry to incorporate them in different ways, tailored to the characteristics of the product and its presentation to consumers in the market.

Considering the physicochemical, organoleptic, and microbiological properties of the product, the protective culture can be added in the formulation, on the surface, or in the packaging.

When added to formulations, protective cultures are included during product development, typically alongside starter cultures. They can be added in various formats: as live bacteria that produce and release antimicrobial compounds during manufacturing, as cell-free supernatant (CFS), acting as an antimicrobial ingredient; in powder or freeze-dried forms; as microencapsulated or spray-dried CFS; or as purified or semi-purified bacteriocins (Adesina & Oluwafemi, 2022; Moradi et al., 2019; Yap et al., 2022).

For some food products sourced directly from nature, bioprotective cultures cannot be added during formulation. Instead, they are applied directly to the food's surface. This can involve spraying, washing, or soaking the product in solutions containing the cultures. Similar to their use in formulations, these protective cultures come in various forms for surface application, including live bacterial cultures, CFS, powdered/lyophilized preparations, or purified bacteriocins (Aymerich et al., 2019; Mostafa, 2020; Vijayakumar & Muriana, 2017).

Traditional methods of incorporating antimicrobial compounds into food have certain drawbacks, such as low diffusion, uneven distribution, and incompatibility with the food matrix or a spectrum of activity that is not suitable for certain foods (da Costa et al., 2019). To overcome these limitations, and in response to the increasing trend of RTE foods and consumer preference for convenience and familiar ingredients (Aschemann-Witzel et al., 2019; Asioli et al., 2017), an innovative approach is emerging using packaging as a carrier for antimicrobial compounds. This strategy involves the incorporation of protective cultures or their components in packaging surfaces or coatings, often including often include thickeners and gelling agents like CMC, glycerol, or konjac (Mohammadi et al., 2022; C. C. Silva et al., 2018). After obtaining the film, protective cultures are added in the following formats: as a lyophile, as pellets or immobilized cells, as encapsulated bacteriocins, or as purified and lyophilized

bacteriocins (Elabd, 2019; Khodaei & Hamidi-Esfahani, 2019; Mohammadi et al., 2022; C. C. Silva et al., 2018; Tumbariski Y., 2019).

It should be noted that the addition of protective cultures is only a part of the barrier technology applied to food, since they are usually accompanied by other techniques such as pasteurization (Vijayakumar & Muriana, 2017), high hydrostatic pressure (Stratakos et al., 2016), light pulses (Pirozzi et al., 2021), active packaging (Elabd, 2019), or modified or controlled atmospheres (Guimaraes et al., 2018), among others.

3. Increasing interest of protective cultures

The application of protective cultures as ingredients to extend the shelf-life of various products has garnered interest for approximately thirty years. However, it is only within the past decade that the food industry has focused on incorporating these cultures for preservation and the development of new products, due to new technologies or new approaches in the preservation of food. This trend is evident in the literature, as indicated by the Scopus database, which shows that the earliest studies on bioprotection in food date to 1995, with the most recent ones published in 2024 (Figure 1).

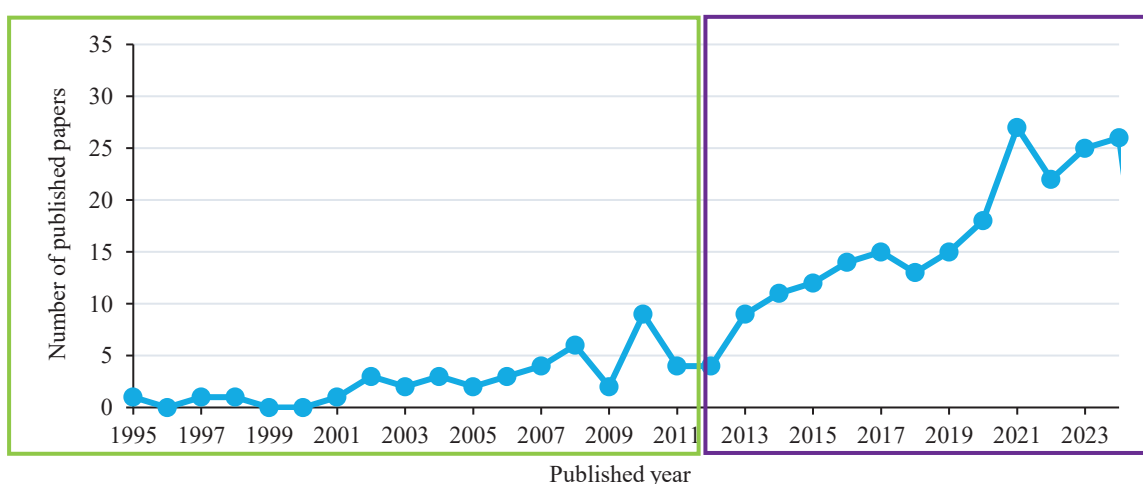


Figure 1. Publications on *bioprotective cultures* (1995 - 2023).

The graph reveals a slow but steady growth in the number of publications from 1995 to 2013. This period of gradual increase suggests an initial phase of exploration and foundational research in the field of protective cultures and their application in the food industry. However, from 2013 onwards, there is a marked acceleration in the growth rate of publications. This indicates a growing interest and development in the field of protective cultures and their application in the food industry. Additionally, this increase may be attributed to regulatory changes, shifts in consumer and industry demands, and the adoption of innovation as an alternative to conventional methods.

On the other hand, it is noteworthy that European countries, including France, Italy, and Spain, have contributed the highest number of publications. This trend underscores the significant interest of the European agrifood industry in these types of ingredients.

In the last decade, scientific publications have systematically included various strategies for incorporating antimicrobial compounds into foods, such as the addition of bacterial cultures, freeze-dried protective cultures, and supernatants. Each of these methods has been the subject of an average of 3-4 scientific publications annually. Figure 2 shows the evolution of the number of studies publishing strategies in addition to protective cultures to foods, highlighting the importance of each method in the scientific literature (Scopus).

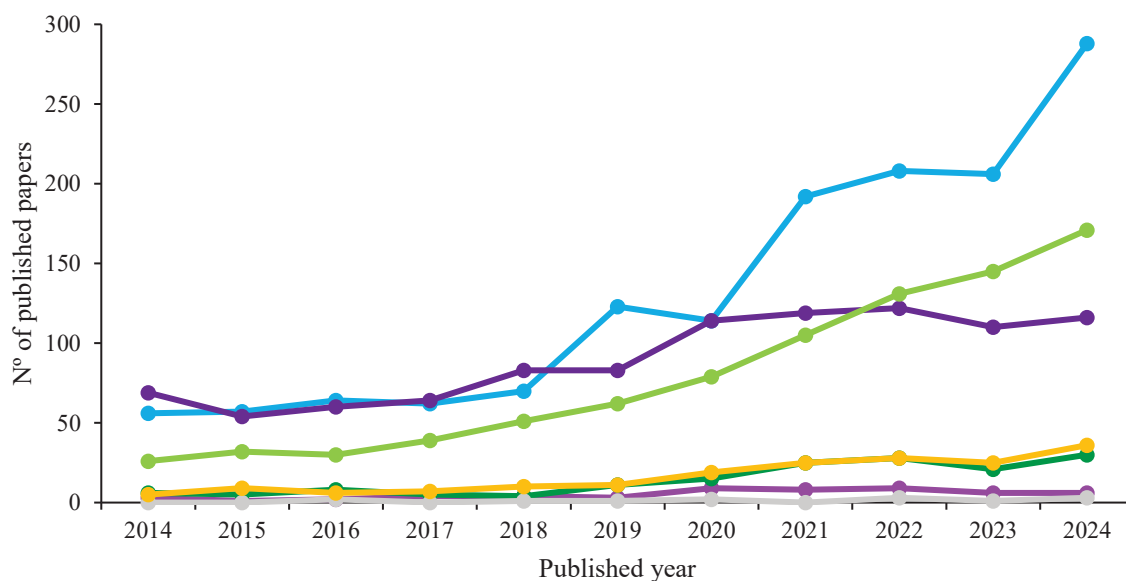


Figure 2. Publications about the addition of protective cultures to foods over the last ten years, depending on the strategy of incorporation: bacterial culture (pink colour), supernatant (dark green), cell free supernatant (light blue), lyophilized (grey), purified bacteriocins (purple), edible coatings (light green), packaging material (orange).

In the last ten years, due to consumer demand and the food industry's offerings, there has been a significant increase in researchers' interest in two strategies: the addition of CFS and the incorporation of purified bacteriocins. In particular, the use of CFS has seen a notable increase, with the number of related publications rising from an annual average of 60 publications (2014-2018) to 180 (2019-2024). Notably, in the periods 2020-2021 and 2023-2024, the total number of publications increased by 150% compared to the previous year. This trend seems to continue in 2025, with 22 publications related to this type of protective culture up to the date of writing this review. This trend suggests that the food industry is increasingly focusing on the application and legal acceptance of these ingredients. On the other hand, the incorporation of purified bacteriocins has historically shown an upward trend, although this seems to have stagnated over the past five years. However, the use of purified bacteriocins is not authorized under European regulations, which could partly explain the recent slowdown in publications describing this approach.

Notably, the strategy of incorporating protective cultures into edible coatings has grown considerably since 2017.

As a summary on the method of application of protective cultures, the method of addition most frequently mentioned in scientific literature, and therefore arguably the most widely used in the food industry, is the cell-free supernatant of bacteria as a protective culture, followed by purified bacteriocins and edible coatings. The specific bacteria used vary depending on the food to which they are added.

4. Current uses of bioprotective cultures in specific foods

Tables 3-7 list some of the trends, according to the matrix, in the use of microorganisms as protective cultures, highlighting the specific products in which these microorganisms are used and the methods of their addition to the food or if they are used with another preservation treatment.

4.1. Fruits and vegetables (Table 3)

Fruits and vegetables are available in various formats in the market, catering to evolving lifestyle and consumer consumption trends. These formats include whole fruits and fresh-cut or RTE options, with the latter gaining prominence due to their convenience. Cut and ready-to-eat fruits are highly perishable; thus, a key objective in this sector of the food industry is to develop preservation techniques that extend the shelf life by inhibiting the growth of pathogens or spoilage microorganisms of these products while maintaining their organoleptic properties. Commonly employed methods include modified atmosphere packaging (MAP) and the addition of additives such as ascorbic acid.

To meet consumer demand for clean label products, an emerging alternative is the incorporation of live bacteria that act as probiotics. These bacteria serve dual roles: acting as antimicrobials and, when labelled as probiotics, providing additional health benefits.

The literature identifies three primary methods for incorporating microorganisms in fruits and vegetables: edible coatings, immersion, and matrix/formulation. Within these methods, microorganisms can be added as cultures or CFS.

Edible coatings are commonly applied to perishable fruits that are cut fresh in various formats or are prone to rapid browning. These coatings also serve as carriers for live bacteria, which can act as probiotics. The literature extensively documents the use of edible coatings on fresh strawberries, with alginate and carboxymethyl cellulose (CMC) being two primary ingredients. Lactic acid bacteria, including *L. plantarum* as pellet (Khodaei & Hamidi-Esfahani, 2019) and *L. rhamnosus* (Li et al., 2024; Temiz & Özdemir, 2021), as well as *Bacillus* spp. (Menéndez-Cañamares et al., 2024; Tumbarski Y., 2019), are incorporated into these formulations effectively inhibiting the growth of moulds and yeasts responsible for spoilage and the reduced shelf life of strawberries. Other products that utilize these techniques include fresh-cut apple (Alvarez et al., 2021; Elabd, 2019; Hashemi & Jafarpour, 2021; Wong et al., 2021), fresh-cut kiwifruit (Hashemi & Jafarpour, 2021), and fresh-cut pineapple (Tenea et al., 2020). The primary microorganisms added to edible coatings in these products are *L. plantarum* and *L. rhamnosus*, in their pellet, culture or CFS format, which serve as protection against pathogens such as *E. coli*, *Listeria* spp., and *Salmonella* spp., as well as spoilage agents like yeasts and moulds.

Another method for incorporating protective cultures or CFS into fruits is immersion, as described for pears (Iglesias et al., 2018), pineapples (Russo et al., 2014), apples (Siroli et al., 2015), peppers (Saravanakumar et al., 2020), or lettuce (Siroli et al., 2015). The CFS of *L. plantarum* is the most used ingredient for immersion, effectively acting against pathogenic microorganisms such as *L. monocytogenes*, *Salmonella* spp., and *E. coli*.

The method of adding protective cultures or CFS to feed varies depending on the product type. Various studies have reported the incorporation of CFS in fruit juices; for instance, Adesina and Oluwafemi (2022) documented the use of CFS from *P. pentosaceus* in orange and watermelon juices to combat the pathogen *S. aureus*. Similarly, Behbahani et al. (2024) added *L. brevis* CFS to peach juice to inhibit the growth of *L. monocytogenes*, *Salmonella* spp., and other spoilage microorganisms. Conversely, for cut fruits such as strawberries (L. Zhao et al., 2022), kiwis (L. Zhao et al., 2022), melon (Abadias et al., 2014), oranges (Chen et al., 2021), and apples (Islam et al., 2023; L. Zhao et al., 2022), the predominant method involves creating a cavity and inoculating both the indicator microorganism and the CP or CFS. In these cases, *L. plantarum* and various strains of *Bacillus* spp. are commonly used to inhibit the growth of molds such as *B. cinerea* or *Penicillium* spp., as well as *L. monocytogenes* and *Salmonella* spp.

4.2. Dairy products (Table 4)

Historically, fermented dairy products have been recognized as fermented due to the addition of starters for fermentation to achieve the final product. Beyond this primary function, the incorporation of other bacteria, acting as probiotics and protective cultures, due to their production of antimicrobial compounds, is being investigated to enhance their organoleptic properties and extend shelf-life.

The most significant proliferation issues in these foods are caused by various species of *Listeria* spp. and lactic acid bacteria, leading to their alteration and deterioration, and posing public health risks.

The incorporation of CP or their CFS into the formulation or matrix is frequently highlighted in scientific literature due to its simplicity during the dairy manufacturing process. Nielsen et al. (2021) demonstrated the use of *L. rhamnosus* in yogurt production

to inhibit the growth of *D. hansenii* yeast. Similarly, (Leyva Salas et al. (2018) and Ouiddir et al. (2019) employed various strains of *L. plantarum* to combat *M. racemosus* in sour cream. Balta & Yerlikaya (2024) successfully inoculated *L. rhamnosus* into kefir formulations, effectively inhibiting the growth of *E. coli* and *E. faecalis*, which are known to cause product spoilage.

L. plantarum is frequently cited in the literature as a key microorganism incorporated into cheese formulations to inhibit the growth of various undesirable microorganisms. For instance, Leyva Salas et al. (2018) utilized *L. plantarum* in semi-hard cheese to inhibit the molds *P. commune* and *M. racemosus*. Additionally, other researchers have employed *L. plantarum* to inhibit *L. monocytogenes* in high-moisture model cheese (Engstrom et al., 2021), laboratory cheese models (Pisano et al., 2022), and Brazilian cheese (Ribeiro et al., 2021). Furthermore, it has been used to inhibit *S. aureus* in domiati-like cheese (Khalil et al., 2022) and brazilian cheese (Ribeiro et al., 2021).

Another technique used to incorporate bacteria or their CFS into cheeses is to wrap them with edible coatings. The formulation of these coatings varies depending on the authors and the type of cheese, achieving the goal of inhibiting pathogenic and/or spoilage microorganisms. In 2019, Guitián et al. tested an agar-based edible coating with the CFS of *E. avium*, which inhibited the growth of *L. monocytogenes* in tybo cheese and handcrafted goat cheese. Other researchers also achieved antimicrobial activity against the same pathogen with different edible coatings. For example, Jutinico-Shubach et al. (2020) tested a glycerol and chitosan-based edible coating formulated with the CFS of *P. pentosaceus* in fresh cheese. Silva et al. (2022) tested in the same product an alginate, maltodextrin, and glycerol-based coating, supplemented with *L. lactis*, and another with *L. garviae*. In other cheeses such as mongolian cheese (Dong et al., 2023) and soft cheese

(El-Sayed et al., 2021), two edible coatings were also tested. One was alginate-based with the CFS of *L. paracasei*, and the other was a combination of chitosan, sodium alginate, and CMC with *B. lactis*, *L. acidophilus*, or *L. casei*, respectively, against pathogenic microorganisms and *Aspergillus* spp. and *Mucor niemalis*.

Finally, de Lima Marques et al. (2017) highlighted the use of biodegradable films formulated with the CFS of *L. curvatus* to be placed between slices of Prato cheese, aiming to inhibit the growth of *Listeria monocytogenes* on the surface and throughout the product. In addition to these types of films, Salvucci et al. (2018) proposed the use of active films incorporating bacteriocin-like substances from the bacterium *E. faecium* to inhibit the proliferation of *L. innocua* in semi-hard white cheese slices.

4.3. Cereals (Table 5)

Cereals serve as the primary ingredient in fermented products, such as bread, where starters are traditionally incorporated into the formulation or sourdough to facilitate fermentation and achieve the desired final product. However, literature reports indicate that the addition of protective cultures or their cell-free supernatants to ingredients or raw materials can inhibit or delay the growth of moulds and yeasts, thereby extending the shelf life of the final products.

The incorporation of these microorganisms primarily occurs as an ingredient in the formulation of sourdough or cereals. The most extensively described microorganisms are LAB, specifically *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei*. These bacteria not only promote fermentation to produce the desired food but also exhibit antimicrobial activity against different spoilage microorganisms. The use of these bacteria against *Aspergillus* spp. has been described by multiple researchers (Ebrahimi et al., 2020; Ouidir et al., 2019; Shehata et al., 2019) in products like bread or wheat

sourdough. Another important mold to combat is *Alternaria* spp., for which Calasso et al. (2023) proposed the use of LAB in pizzas and focaccias.

Other methods of application have been described, such as the spraying of two combinations of LAB onto a cereal based dessert known as milk bread rolls, as detailed by Le Lay et al. (2016), which showed that way of addition as a viable alternative for inhibiting the growth of spoilage microorganisms such as *Eurotium repens*, *Aspergillus niger* and *Penicillium corylophilum*.

Additionally, Gregirchak et al. (2020) proposed the use of edible coatings formulated with various LAB, sodium alginate, yeast, and glycerol to combat moulds such as *Aspergillus* spp. and *Penicillium* spp. in wheat bread.

In the case of seeds, not only are studies being developed in which the inoculation of PC or CFS is carried out, but in 2021, Somashekaraiah et al. proposed, with positive results, the immersion of maize seeds in the CFS of *L. brevis* MYSN105 at 16% to inhibit the growth of pathogenic microorganisms such as *E. coli*, *S. aureus*, and *Salmonella* spp. that commonly appear in these ingredients.

4.4. Meat products (Table 6)

Traditionally, lactic acid bacteria have been incorporated into various meat formulations to produce fermented products. However, their use has evolved, and they are now being utilized as alternatives to artificial preservatives with E-numbers.

The most described methods for incorporating these bacteria into meat include dipping, addition to the matrix or formulation, and surface inoculation. For dipping, strains of LAB species such as *L. paracasei* (Duan et al., 2020), *L. sakei* and *L. curvatus* (Zhang et al., 2017), or *P. acidilactici* (İncili et al., 2023) have been used on fresh chicken breast to combat *Pseudomonas* spp., *Hafnia alvei*, or *Serratia* spp.; on refrigerated beef to inhibit the growth of *Enterobacteriaceae*, *Pseudomonas* spp., and *B.*

thermosphacta; and on frankfurters, respectively. Additionally, *C. maltaromaticum* has been proposed by de Andrade Cavalari et al. (2024) as a potential protective culture for sliced cooked ham.

Surface inoculation involves the addition and spreading of bacteria using an L-shaped seeding loop. Barcenilla et al. (2023) highlighted the effectiveness of this method in fresh cooked ham slices, meatloaf, and roasted pork, using *L. lactis*, *L. plantarum*, and *L. paracasei* bacteria, which showed positive results against *L. monocytogenes*. Additionally, Bungenstock et al. (2021) demonstrated the antimicrobial activity of surface inoculation with *Pediococcus* spp. against *L. monocytogenes*.

The incorporation of these bacteria has also been achieved by inoculating them into the formulation or matrix of the final product. This method aims to inhibit the growth of pathogenic bacteria such as *L. monocytogenes* or *E. coli* in fresh raw top round beef by adding *L. plantarum* to the marinade (Arrioja-Bretón et al., 2020). Other bacteria, such as *P. acidilactici*, *L. sakei*, and *Staphylococcus* spp., have shown positive effects on shelf-life extension in beef hamburgers by inhibiting the growth of *B. thermosphacta* (Comi et al., 2015) and in Sardinian fermented sausages by inhibiting the growth of *L. monocytogenes* (Siddi et al., 2022). Additionally, *C. maltaromaticum* can be added in this format to inhibit the growth of *Pseudomonas fluorescens* and *Bacillus thermosphacta* (de Andrade Cavalari et al., 2024).

In addition to these conventional techniques, more innovative methods such as the use of active films and edible coatings as carriers of probiotics have been explored. These carriers act as protective cultures due to their antimicrobial activity. This type of film or plastic has been validated in sliced meat products; for instance, Settler-Ramírez et al. (2021) and Pereira et al. (2018) added *L. lactis*, *L. casei*, and *B. animalis* cultures to sliced ham in active film and edible coating formats, respectively, to inhibit the growth of

pathogenic microorganisms such as *L. monocytogenes* and *Pseudomonas* spp.. Additionally, the incorporation of *L. plantarum* into the film has been noted for its ability to inhibit *L. monocytogenes* in fresh ground beef (Yordshahi et al., 2020).

4.5. Seafood products (Table 7)

Contemporary seafood products have evolved to meet consumer demands for clean labels, convenience, and rapid consumption. Consequently, ready-to-eat seafood items frequently encounter *Listeria monocytogenes* contamination, posing significant food safety concerns.

To inhibit the growth of both pathogenic and spoilage microorganisms while providing clean label products, the incorporation of lactic acid bacteria and/or their metabolites is a viable alternative supported by the literature.

Various methods for inoculating these microorganisms into fish or fish by-products have been described, with surface spraying being one of the most extensively studied techniques. For instance, Sánchez-Martín et al. (2024) highlighted the use of *L. carnosus* and *L. lactis* as protective cultures against different strains of *L. monocytogenes* in cold-smoked rainbow trout. Similarly, Anacarso et al. (2014), Gómez-Sala et al. (2016), and Bolívar et al. (2021) reported positive results using different LAB strains, including *L. pentosus* in fresh salmon filets, *L. curvatus* in young hake and megrim, and *L. sakei* in surimi-based product and tuna paté, respectively, against *L. monocytogenes*. Other researchers have successfully inhibited the growth of various microorganisms. For example, Leroi et al. (2015) focused on *S. proteamaculans* and *Brochothrix thermosphacta* in cold-smoked salmon, using *L. piscium* bacteria to combat them. Similarly, Saraoui et al. (2017) inhibited the growth of *S. proteamaculans* in cooked and peeled shrimps by using *L. piscium* and *C. divergens*. As shown in the table, other authors have also achieved positive results working with different products and microorganisms.

Another technique developed in these studies involves immersing the product in a broth containing protective cultures or their CFS. Several authors have highlighted this methodology in cold-smoked fish to inhibit the growth of *L. monocytogenes* using *L. sakei*, *L. curvatus* and *C. maltaromaticum* (Aymerich et al., 2019), *Carnobacterium* spp. (Iacumin et al., 2021), *Leuconostoc* spp. and *Weisella* spp. The latter was also applied to two other products, gravlax and sushi (Stupar et al., 2021).

Finally, Bolívar et al. (2021) described a technique involving the use of a sterile syringe to inoculate the lactic acid bacteria *L. sakei* into tuna pate, effectively inhibiting the growth of *L. monocytogenes*.

Table 3. List of publications on bioprotective cultures applied in fruits and vegetables, according to different strategies of incorporation.

Edible coatings					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Fresh-cut apple slices	<i>Lactocaseibacillus rhamnosus</i> <i>Bifidobacterium animalis</i> subsp. <i>lactis</i>	<i>Listeria innocua</i> CIP 80.11 and <i>Escherichia coli</i> O157:H7 FP605/03	Alginate-based edible coating + polypropylene containers with a polyolefine film	5±1°C for 8 days (in darkness)	Álvarez et al., 2020
Cherry tomatoes	<i>Lactiplantibacillus plantarum</i> A6	<i>Fusarium</i> spp. and <i>Rhizopus stolonifer</i>	Exopolysaccharide (<i>Weissella confusa</i> JCA4)-based coating + PET packages	4°C for 8 days/ 30°C for 8 days	Álvarez et al., 2021
Fresh-cut apple slices	<i>Lactocaseibacillus rhamnosus</i> B-445	Aerobic mesophiles, and yeasts and moulds	Alginate-based edible coating + packaging	5±1°C for 13 days (70-75% relative humidity)	Elabd, 2019
Table grapes	<i>Lactiplantibacillus plantarum</i> 11-A <i>Lactiplantibacillus plantarum</i> LP3 <i>Lactiplantibacillus plantarum</i> LU5 <i>Lactiplantibacillus plantarum</i> AF1	<i>Aspergillus niger</i> CECT 2805	Alginate-based edible coating + plastic containers under passive atmosphere	4°C for 14 days	de Simone et al., 2024
Fresh-cut kiwis		Yeast and moulds	Konjac-based edible coating + polyethylene terephthalate	4°C for 5 days	Hashemi and Jafapour, 2021
Avocado	<i>Meyerozyma caribbica</i>	<i>Colletotrichum gloeosporioides</i> Pa14	Sodium alginate-based edible coating	25 °C for 15 days (75 % RH)/ 6°C (95 % RH) for 10 days and ripened at 25°C (75 % RH) for 5 days	Iníiguez-Moreno et al., 2020
Fresh strawberries	<i>Lactiplantibacillus plantarum</i>	Yeast and moulds	Carboxymethyl Cellulose (CMC) edible coating	4°C for 16 days	Khoadei and Hamidi-Esfahani, 2019
Strawberries	<i>Lactocaseibacillus rhamnosus</i> GG (LGG) CICC 6224	<i>Escherichia coli</i> O157:H7 9637 <i>Staphylococcus aureus</i>	Alginate-based edible coating + polypropylene containers	28°C for 8 days (80±2% relative humidity)	Li et al., 2024
Strawberries	<i>Bacillus subtilis</i> SB8	<i>Botrytis cinerea</i>	Sodium-alginate and glycerol based edible coating + sterilized food containers with damp paper	Room temperature for 5 days (in darkness)	Menéndez-Canamares et al., 2024
Mushrooms cream	<i>Lactococcus lactis</i> subsp. <i>lactis</i> CECT 539/ ATCC 11454	<i>Listeria monocytogenes</i> CECT 934/ ATCC 19114	(PVOH + Hgel) films + thermo-sealed bags	4°C for 15 days	Settier-Ramírez et al., 2021
Carrots sticks	<i>Lactobacillus acidophilus</i> La-14	Aerobic mesophiles, and yeasts and moulds	Sodium-alginate based edible coating + polystyrene trays sealed with vinyl polychloride film	8±2°C for 19 days	Shigematsu et al., 2018
Fresh strawberries	<i>Lactocaseibacillus rhamnosus</i>	Aerobic mesophiles, and yeasts and moulds	Gelatin-based edible coating Gelatin-inulin based edible coating	4°C for 16 days	Temiz and Özdemir, 2021

Table 3 (continuation)

Edible coatings (continuation)					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Pineapple	<i>Lactiplantibacillus plantarum</i> UTNCys5-4 and <i>Lactococcus lactis</i> subsp. <i>lactis</i> Gt28	<i>Escherichia coli</i> ATCC25922, <i>Escherichia coli</i> UTNEc1, <i>Salmonella enterica</i> subsp. <i>enterica</i> ATCC51741, <i>Salmonella</i> UTNSm2 and <i>Shigella sonnei</i> ATCC25931	CFS peptide-based formulation	Refrigerated temperature for 5 days	Tenea et al., 2020
Fresh strawberries	<i>Bacillus methylotrophicus</i> BM47	Yeast and moulds	Carboxymethyl Cellulose (CMC) edible coating + plastic bags	4°C for 16 days (75% relative humidity)	Tumbariski et al., 2019
Blackberries	Lactic acid (LA-17.5)	<i>Mucor racemosus</i>	Chitosan-based edible coating	4°C for 14 days	Vilaplana et al., 2020
Fresh-cut apple slices	<i>Lactiplantibacillus plantarum</i> 299v	<i>Listeria monocytogenes</i> BAA-839	Carboxymethyl Cellulose (CMC) edible coating (1st coating) + zein coating (2nd coating) + ppolypropylene fresh-keeping boxes	4°C for 7 days	Wong et al., 2021
Fresh pistachio	<i>Lactocaseibacillus casei</i> XN18	Yeast and moulds	<i>Lallemanitia royleana</i> mucilage based edible coating + polyehylene plastic bags	4°C for 35 days (85% relative humidity)	Zibaei-Rad et al., 2024
Immersion					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Hazelnuts	<i>Metchnikowia</i> aff. <i>pulcherrima</i> DN-HS	<i>Aspergillus flavus</i>		25°C for 7 days	Dikmetas et al., 2023
Cut iceberg lettuce	<i>Lactococcus lactis</i> 537	<i>Listeria monocytogenes</i> ATCC 7644 <i>Listeria monocytogenes</i> CECT 4031 (serovar 1a), CECT 933 (serovar 3a), CECT 940 (serovar 4d), CECT 4032 (serovar 4b) and serovar 1/2a	Commercial plastic zip lock bags	4°C for 10 days	Dong et al., 2021
Fresh-cut pear	<i>Lactocaseibacillus rhamnosus</i> GG (LGG)	<i>Salmonella enterica</i> subsp. <i>enterica</i> ATCC BAA-707, ATCC BAA-709 and CECT 4300	Polypropylene trays sealed with non-peelable polypropylene plastic film	5°C for 9 days	Iglesias et al., 2018
Fresh-cut pear	<i>Pseudomonas graminis</i> CPA-7	<i>Listeria monocytogenes</i> CECT 4031 (serovar 1a), CECT 933 (serovar 3a), CECT 940 (serovar 4d), CECT 4032 (serovar 4b) and 1/2a	Antioxidant solution (2% ascorbic acid + 2% sodium citrate + 1% CaCl2) and MAP	5°C for 10 days/ 10°C for 10 days	Iglesias et al., 2018
Pineapple	<i>Lactobacillus plantarum</i> B2 (CECT 8328) <i>Lactobacillus fermentum</i> PBCC11.5 (CECT 8448)	<i>Listeria monocytogenes</i> CECT 4031 and <i>Escherichia coli</i> O157:H7 CECT 4267	Polypropylene plastic film bags with thermally sealed in MAP	5°C for 8 days	Russo et al., 2014
Red and yellow fresh-cut bell pepper	<i>Lactocaseibacillus rhamnosus</i> GG (LGG) (ATCC 53103)	<i>Listeria monocytogenes</i> ATCC 19118 <i>Salmonella</i> Typhimurium ATCC 14028	Biochemical additive solution (sodium benzoate + ascorbic acid + DL- α -tocopherol acetate) + polypropylene container	4°C for 15 days/ 15°C for 15 days	Saravanakumar et al., 2020

Table 3 (continuation)				
Immersion (continuation)				
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Apple slices	<i>Lactiplantibacillus plantarum</i> CIT3	<i>Escherichia coli</i> , <i>Salmonella</i> spp. and <i>Listeria monocytogenes</i>	2-(E)-hexenal/ hexanal and citral/2-(E)-hexenal + active modified atmosphere (7% O ₂ / 0% CO ₂)	6°C for 16 days
Lamb's lettuce	<i>Lactiplantibacillus plantarum</i> V7B3		Thyme essential oil + artificial ordinary atmosphere	Siroli et al., 2014
Matrix/formulation				
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Fresh-cut melon	<i>Pseudomonas graminis</i> CPA-7	<i>Salmonella enterica</i> subsp. <i>enterica</i> ATCC BAA-709, ATCC BAA-710 and ATCC BAA-711 <i>Listeria monocytogenes</i> CECT 4031 (serovar 1a), CECT 933 (serovar 3a), CECT 940 (serovar 4d), CECT 4032 (serovar 4b) and serovar 1/2a	Passive MAP with polypropylene trays sealed with a non peelable polypropylene plastic film/ air packaging with non peelable polypropylene plastic film with ten holes of 400µm	5°C for 8 days/ 10°C for 8 days
Water-melon juices	<i>Pediococcus pentosaceus</i> IO1	<i>Staphylococcus aureus</i>	Sterilized glass bottles	Room temperature for 72 hours
Orange juices				Adesina and Oluwafemi, 2022
Cherries	<i>Bacillus subtilis</i> Y17B	<i>Alternaria alternata</i> ACT-1	Sterilized six-well culture cluster flat-bottom plates and covered with a zipper bag	28°C for 7 days
Peach juice				Ahmad et al., 2023
	<i>Levilactobacillus brevis</i> HL6	<i>Staphylococcus aureus</i> <i>Listeria monocytogenes</i> <i>Salmonella enterica</i> serovar Typhimurium <i>Shigella dysenteriae</i> <i>Bacillus cereus</i> <i>Streptococcus pyogenes</i>		4°C for 21 days/ 25°C for 21 days
Orange fruit	<i>Lactiplantibacillus plantarum</i> CKXP13 and CWXP24	<i>Penicillium digitatum</i>	Plastic bags	25°C for X days (high relative humidity)
Apple juice	<i>Ralstonia</i> spp. SL312	Patulin (<i>Penicillium</i> spp., <i>Aspergillus</i> spp. y <i>Byssoschlamys</i> spp.)		37°C for 32 hours
Fresh-cut apple				He et al., 2022
Grapes	<i>Lactiplantibacillus plantarum</i> DMR14	<i>Shigella boydii</i>		37°C for 48 hours
Banana				Islam et al., 2023
Sugarcane juice	<i>Wickerhamomyces anomalus</i>	<i>Botrytis cinerea</i>	Plastic baskets and wrapped with cling film	20°C for 4 days (95% relative humidity)
Tomatoes		<i>Listeria monocytogenes</i> ATCC 19112		5°C for 21 days/ 10°C for 21 days/ 15°C for 21 days
Cream of potato / meat soups	<i>Lactocaseibacillus rhamnosus</i> GG (LGG)	<i>Salmonella</i> Typhimurium ATCC 13311	Hot-filled into multi-layered plastic pouches sealed with the time knob at 6	Muñoz et al., 2019

Table 3 (continuation)

Matrix/formulation (continuation)		Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Ready-to-eat salads	<i>Weissella viridescens</i>		<i>Listeria monocytogenes</i> LR102 (serotype 1/2a), VI 51028 (serotype 4), 0227e359 (serotype 1), 0113e131 (serotype 1) and VI 51010 (serotype 4b)	High Hydrostatic Pressure (HHP) (400 MPa/1 min) + packaged using polyethylene/polyamide vacuum pouches	4°C for 21 days/ 12°C for 21 days	Stratakos et al., 2016
Cherry tomatoes						
Fresh-cut apple						
Fresh-cut kiwi		<i>Bacillus velezensis</i> A4	<i>Botrytis cinerea</i>	Air dried	25°C (95% relative humidity)	Zhao et al., 2022
Fresh-cut strawberries						

Table 4. List of publications on bioprotective cultures applied in dairy products, according to different strategies of incorporation.

Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
White semi-firm cheese slices	<i>Enterococcus faecium</i> ES216	<i>Listeria innocua</i> ATCC 33090	Triticale flour film + bacteriocin like substance (BLIS)	7°C for 15 days	Salvucci et al., 2018
Biodegradable films					
Sliced Prato cheese	<i>Lactobacillus curvatus</i> p99	<i>Listeria monocytogenes</i> Scott A	Filmogenic solution with starch, distilled water, glycerol, Tween 20	4°C for 10 days	Marques et al., 2017
Edible coating					
Sliced cheese	<i>Lactocaseibacillus rhamnosus</i>	<i>Candida albicans</i> CICC 32819 <i>Zygosaccharomyces rouxii</i> LJL117 <i>Rhodotorula glutinis</i> CICC 33037 <i>Penicillium</i> spp. <i>Mucor niemalis</i> CICC 40868 <i>Aspergillus</i> spp. <i>Staphylococcus aureus</i> 8325 <i>Salmonella Typhimurium</i> 14028s <i>Listeria monocytogenes</i> 598 <i>Escherichia coli</i> O157:H7 9637 <i>Bacillus cereus</i> B-3711 <i>Aspergillus</i> spp.	Sodium caseinate, glycerol-based edible coatings	4°C for 30 days	Ceylan and Atasoy, 2023
Mongolian cheese	<i>Lactocaseibacillus paracasei</i> ALAC-4	<i>L. monocytogenes</i> 01/155	Alginate-based edible coating	4°C for 15 days	Dong et al., 2023
Soft cheese	<i>Bifidobacterium lactis</i> , <i>Lactobacillus acidophilus</i> and <i>Lactocaseibacillus casei</i>	<i>Listeria monocytogenes</i> ATCC 7644	Chitosan, sodium alginate and carboxymethyl cellulose (CMC)-based edible coating	7°C for 45 days	El-Sayed et al., 2021
Tybo cheese	<i>Enterococcus avium</i> DSMZ 17511		Agar-based (0.8% w/v) edible coating	4°C for 14 days/ 8°C for 14 days	Guitián et al., 2019
Handcraft goat cheese	<i>Pediococcus pentosaceus</i> 147		Glycerol and chitosan-based edible coatings + individual aseptic bags	4°C for 21 days	Jutinico-Shubach et al., 2020
Fresh cheese	<i>Lactobacillus lactis</i> L3A21M1 <i>Lactococcus garvieae</i> SIC17		Alginate, maltodextrin and glycerol-based edible coating	4°C for 10 days/ 10°C for 10 days	Silva et al., 2022
Fresh-cheese					
Artisanal acid-curd cheese	<i>Lactobacillus helveticus</i> MI-LH13	Yeasts Moulds <i>Pseudomonas</i> spp. <i>Clostridium</i> spp.	Glycerol-based edible coating	4±1°C for 14 days/ 23±1°C for 14 days	Vasiliauskaite et al., 2022

Table 4 (continuation)

Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Fresh cheese	<i>Lactocaseibacillus</i> <i>rhamnosus</i> LRH05	<i>Pseudomonas</i> spp. and <i>Enterobacteriaceae</i>	Modified atmosphere (MAP) (70% N ₂ / 30% CO ₂)	8 for 5 weeks/ 14°C for 5 weeks	Bassi et al., 2020
	<i>Lactilactobacillus sakei</i> LSK04				
	<i>Carnobacterium</i> <i>maltaromaticum</i> CNB06				
Kefir	<i>Lactocaseibacillus</i> <i>rhamnosus</i>	<i>Escherichia coli</i> ATCC 25922 and <i>Enterococcus faecalis</i> 29212	200mL packages	4°C for 21 days	Balta and Yerlikaya, 2024
	<i>Propionibacterium</i> <i>freudenreichii</i> subsp. <i>shermanii</i>				
	<i>Enterococcus faecalis</i>				
Fresh-cheesse		<i>Listeria monocytogenes</i> ATCC 7644	Sterile stain steel circular cheese containers and mesh covered	4°C for 15 days	Coelho et al., 2014
Sheep cheese whey	<i>Lactocaseibacillus casei</i>	Total psychrotrophic bacteria count and total bacteria count		7°C for 28 days	dos Santos et al., 2024
Goat cheese whey		<i>Listeria monocytogenes</i> LM 108 (serovar 1/2b), LM 301 (serovar 1/2a), LM 310 (serovar 4b), R2-500 (serovar 4b) and R2-501 (serovar 4b)	EVOH plastic + vacuum	4 ± 0.5°C for 8 to 10 weeks	Engstrom et al., 2021
High-moisture model cheese	<i>Lactiplantibacillus</i> <i>plantarum</i>				
Cottage cheese	<i>Lactocaseibacillus</i> <i>rhamnosus</i> (+ <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> A026)	<i>Penicillium chrysogenum</i> LMA-212		6°C for 21 days (in darkness)	Fernández et al., 2017
Sheep cheese whey	<i>Lactocaseibacillus casei</i> BGP1-304799A	Psychrotrophic bacteria		7°C for 28 days	Junior and Tribst, 2022
Goat cheese whey	<i>Lactiplantibacillus</i> <i>plantarum</i> FFNL1810				
Domiat-like cheese	<i>Lactiplantibacillus</i> <i>plantarum</i> FFNL739	<i>Staphylococcus aureus</i> ATCC 6538		6 ± 1°C for 56 days	Khalil et al., 2021
	<i>Lactocaseibacillus casei</i> 116			35 days at 6°C and 90% relative humidity (RH), 10 days at 8°C and 80% RH, 10 days at 9°C and 80% RH, and 35 days at 10°C and 80% RH	Martin et al., 2022
Torta del Casar cheese	<i>Lactococcus garvieae</i> 151	<i>Listeria monocytogenes</i>		10°C for 2 weeks	Mieszkina et al., 2017
Fermented milk	<i>Schleiferilactobacillus</i> <i>harbinensis</i> K. V9.3.1Np	<i>Yarrowia lipolytica</i> UBOCC-A- 211004			

Table 4 (continuation)

Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Yogurt	<i>Lactocaseibacillus rhamnosus</i>	<i>Debaryomyces hansenii</i> CHCC 16374 <i>Yarrowia lipolytica</i> CHCC 16375 <i>Saccharomyces cerevisiae</i> CHCC 16590 <i>Kluyveromyces marxianus</i> CHCC 16601	Microtiter with a breathable lid	7°C for 31 days/ 12°C for 31 days and 16°C for 31 days	Nielsen et al., 2020
	<i>Lactiplantibacillus plantarum</i> CH1 <i>Lactococcus lactis</i> 16FS16-9/20234 - 11FS16	<i>Mucor racemosus</i> <i>Penicillium commune</i>	Sterile containers	Incubation at 25°C for 5-10 days	Ouiddir et al., 2019
	<i>Lactiplantibacillus plantarum</i> 1/14537 - 4A/20045	<i>Listeria monocytogenes</i> ATCC 7644	Plastic container	10°C for 10 days (85% humidity)	Pisano et al., 2022
Brazilian cheese	<i>Lactiplantibacillus paraplantarum</i> FT-259 <i>Lactococcus lactis</i> QMF 11	<i>Listeria monocytogenes</i> ATCC 7644 <i>Staphylococcus aureus</i> ATCC 25923	Polyethylene packaging	8°C for 21 days	Ribeiro et al., 2021
Tomato cheese spreads	<i>Latilactobacillus sakei</i> subsp. <i>sakei</i> 2a <i>Lactobacillus plantarum</i> L244 +	<i>Listeria monocytogenes</i> serovar 4b	Plastic vials for food products	4°C for 28 days/ 15°C for 28 days	Ruiz-Martínez et al., 2015
Sour cream	<i>Schleiferilactobacillus harbinensis</i> L172 <i>Lactobacillus plantarum</i> L244 + <i>Lactocaseibacillus rhamnosus</i> CIRM-BIA1113 <i>Lactobacillus plantarum</i> L244 +	<i>Penicillium commune</i> UBOCC-A-116003/ <i>Mucor racemosus</i> UBOCC-A-116002/ <i>Rhodotorula mucilaginosa</i> UBOCC-A-216004	Refrigeration for 24 days		Salas et al., 2018
Semi-hard cheese	<i>Schleiferilactobacillus harbinensis</i> L172 <i>Lactobacillus plantarum</i> L244 + <i>Lactocaseibacillus rhamnosus</i> CIRM-BIA1113	<i>Penicillium commune</i> UBOCC-A-116003/ <i>Mucor racemosus</i> UBOCC-A-116002		12°C for 24 weeks (96% relative humidity)	

Table 4 (continuation)

Matrix/formulation (continuation)					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Crescenza cheese	<i>Penicillium csaseifuvulm</i> FUA 5008 and <i>Penicillium roqueforti</i> 3969	<i>Lactcaseibacillus rhamnosus</i> FUA3185 <i>Lactcaseibacillus paracasei</i> FUA3413		9°C for 1 week, 13°C for 3 weeks, 9°C for 4 weeks and 5°C until 90 days	Zhao et al., 2024
	<i>Debaryomyces hansenii</i> FUA4064	<i>Lactiplantibacillus plantarum</i> FUA 3183 <i>Lactiplantibacillus plantarum</i> FUA 3247			
Whey beverages	<i>Lactiplantibacillus plantarum</i> N7	<i>P. pastoris</i> , <i>A. niger</i> , <i>G. candidum</i> , <i>K. marxianus</i> and <i>P. chrysogenum</i>		4±2°C for 10 days/ room temperature dor 10 days	Xu et al., 2021
Sprayed					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Ricotta fresca cheese	<i>Carnobacterium</i> spp.	<i>Pseudomonas</i> spp.	Modified atmosphere (MAP) (70% N2/ 30% CO2)	4°C for 21 days/ 10°C for 10 days	Spanu et al., 2018
Surface inoculation					
Food	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Cottage cheese	<i>Lactiplantibacillus plantarum</i>	<i>Penicillium commune</i>		Room temperature for 29 days	Cheong et al., 2014

Table 5. List of publications on bioprotective cultures applied in cereals, according to different strategies of incorporation.

Edible coating				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Wheat bread	<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> and <i>Lactobacillus acidophilus</i>	<i>Aspergillus</i> spp. and <i>Penicillium</i> spp.	Sodium alginate, whey and glycerol-based edible coating	23 ± 2°C for 120 hours (relative humidity of 75 ± 5%)
Immersión				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Maize seeds	<i>Levilactobacillus brevis</i> MYSN105	<i>Fusarium verticillioides</i> <i>Escherichia coli</i> ATCC 25922, <i>Staphylococcus aureus</i> ATCC 6538, and <i>Salmonella Paratyphi</i> ATCC 9150	Sterile petri dishes	28°C for 7 days
Matrix/ formulation				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Fresh based-pizza	<i>Lactobacillus acidophilus</i> , <i>Lactocaseibacillus casei</i> , <i>Bifidobacterium</i> spp. and <i>Bacillus coagulans</i>	<i>Alternaria infectoria</i> <i>Alternaria alternata</i>	Modified Atmosphere Packaging (MAP) (50% CO ₂ / 50% N ₂)	4°C for 70 days
Focaccia	<i>Pediococcus pentosaceus</i>	<i>Aspergillus niger</i> ATCC 9029		Room temperature (25°C) for 7 days
Bread	<i>Lactobacillus acidophilus</i> , <i>Bifidobacterium animalis</i> , <i>Lactocaseibacillus paracasei</i> , <i>Lactocaseibacillus casei</i> and <i>Bacillus coagulans</i>	Aerobic mesophilic m.o. at 30°C/ Mesophilic LAB, yeasts and moulds	Modified Atmosphere Packaging (MAP) (40% CO ₂ / 60% N ₂)	4 ± 2°C for 120 days
Fresh pasta				Ebrahimi et al., 2020 Marzano et al., 2022
Bread	<i>Lactiplantibacillus plantarum</i> CH1	<i>Aspergillus tubingensis</i> <i>Aspergillus flavus</i> <i>Aspergillus niger</i> ATCC 9029 (aflatoxins)		Incubation at 25°C for 5-10 days Ouidir et al., 2019
Wheat sourdough	<i>Limosilactobacillus reuteri</i>			Room temperature (25°C) for 7 days Sadeghi et al., 2018
Wheat grains	<i>Lactobacillus</i> sp. RM1	<i>Aspergillus parasiticus</i> ITEM 11	Sterilized grinder	28°C for 15 day Shehata et al., 2019
Cereal-based, potentially probioticand/or synbiotic fermented vegan product (vegan dessert)	<i>Lactiplantibacillus plantarum</i> O21	<i>Enterobacteriaceae</i> , <i>Escherichia coli</i> , coliforms, yeasts and moulds		4°C for 21 days Szydłowska et al., 2021

Table 5 (continuation)

Matrix/ formulation (continuation)					
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Fava bean and wheat bread	<i>Levilactobacillus brevis</i> AM7	<i>Penicillium paneum</i> , <i>Penicillium roqueforti</i> DPPMAF1, and <i>PI</i> , <i>Penicillium crustosum</i> , and <i>Penicillium albocoremium</i> , <i>Eurotium herbariorum</i> <i>Aspergillus niger</i>	Polyethylene bags	Room temperature for 15 days	Verni et al., 2023
Sprayed					
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Cake and milk bread rolls	<i>Leuconostoc citreum</i> L123 and L32, <i>Latilactobacillus sakei</i> O25, <i>L</i> <i>imosilactobacillus reuteri</i> 5529 and <i>Levilactobacillus brevis</i> Lu 35	<i>Cladosporium sphaerospermum</i> UBOCC-A-112116 + <i>Wallemia sebi</i> UBOCC-A-11209 <i>Eurotium repens</i> UBOCC-A-112075 + <i>Aspergillus niger</i> UBOCC-A- 112064 + <i>Penicillium corylophilum</i> UBOCC-A-112081		25°C for 1 week (humid environment)	Le Lay et al., 2016

Table 6. List of publications on bioprotective cultures applied in meat products, according to different strategies of incorporation.

Active films				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Fresh ground beef	<i>Lactiplantibacillus plantarum</i> subsp. <i>Plantarum</i> ATCC 14917	<i>Listeria monocytogenes</i> ATCC 19115	Polyethylene containers and BNC film	4 ± 1°C for 9 days
Sliced cooked ham	<i>Lactococcus lactis</i> subsp. <i>Lactis</i> CECT 539/ ATCC 11454	<i>Listeria monocytogenes</i> CECT 934/ ATCC 19114	PLA/(PVOH + Hgel) films	4°C for 15 days
Edible coatings				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Lamb meat slices	<i>Lactococcus lactis</i> NJ414	Total viable count and psychrotrophic bacteria	Plantago major mucilage (PMM)	4°C for 9 days
Sliced ham	<i>Bifidobacterium animalis</i> Bb-12 <i>Lactocaseibacillus casei</i> -01	<i>Staphylococcus</i> spp., <i>Pseudomonas</i> spp., <i>Enterobacteriaceae</i> and <i>yeasts/moulds</i>	Whey protein and glycerol-based edible coating	4°C for 45 days
Immersion				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Sliced cooked ham	<i>Carnobacterium maltaromaticum</i> CM_B824, CM_B827 and CM_B289	<i>Pseudomonas fluorescens</i> ATCC 1355 and <i>Bacillus thermosphacta</i> ATCC 11509 + indigenous	PP/EVOH/PPR trays with modified atmosphere (70% N ₂ / 30% CO ₂) sealed with PET/PP film	4°C for 10 days and 8°C for 18 days
Fresh chicken breast	<i>Lactocaseibacillus paracasei</i> FX-6	<i>Pseudomonas</i> spp. <i>Hafnia-Obesumbacterium</i> <i>Serratia</i> spp.	Sterile petri dishes covered with parafilm	4°C for 14 days
Chilled beef	<i>Latilactobacillus sakei</i> (Bactoferm B-2) <i>Latilactobacillus curvatus</i> (SafePro B-LC-48)	<i>Enterobacteriaceae</i> , <i>Pseudomonas</i> spp. and <i>B. thermosphacta</i>	Vacuum bags were composed of polyamide/ polyethylene film	4°C for 38 days
Frankfurters	<i>Pediococcus acidilactici</i> B-LC-20	Total viable count <i>Escherichia coli</i> <i>Salmonella</i> Typhimurium <i>Listeria monocytogenes</i> <i>Staphylococcus aureus</i> ATCC 29213 and ATCC 25923	Vacuum-packaged at -1 bar vacuum level a	4±1°C for 28 days
Fresh raw top round beef	<i>Lactiplantibacillus plantarum</i> NRRL B-4496		Marinade	4°C for 14 hours
Ground beef	<i>Carnobacterium maltaromaticum</i> CM_B824, CM_B827 and CM_B289	<i>Pseudomonas fluorescens</i> ATCC 1355 and <i>Bacillus thermosphacta</i> ATCC 11509 + indigenous	PP/EVOH/PPR trays with modified atmosphere (66% O ₂ / 4% N ₂ , 30% CO ₂) sealed with PET/PP film	4°C for 3 days and 8°C for 4 days (0% relative humidity)

Table 6 (continuation)

Matrix/ formulation						
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author	
Beef hamburgers	<i>Latilactobacillus sakei</i> subsp. <i>carnosus</i> / <i>Latilactobacillus sakei</i> + <i>Staphylococcus xylosum</i>	<i>Brochothrix thermosphacta</i>	Modified atmosphere packaging (70% N2/ 30% CO2) + PET/PE/EVOH/PE/ANTIFOG - EVOH trays laminated with a film consisting of PET/PE/EVOH/PE/ANTIFOG - EVOH	4±2°C for 12 days	Comi et al., 2015	
	<i>Latilactobacillus sakei</i> , <i>Pediococcus acidilactici</i> , <i>Staphylococcus carnosus</i> and <i>Staphylococcus carnosus</i> subsp. <i>utilis</i>	<i>Listeria monocytogenes</i>	Nitrate and nitrite, ascorbic acid	12 - 15°C for 20 days (70 - 75% humidity)	Sidi et al., 2022	
	<i>Pediococcus acidilactici</i>					
Sprayed						
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author	
Chicken sausages	<i>Latilactobacillus sakei</i> B-2 <i>Latilactobacillus curvatus</i> B-LC-48	Psychrotrophic bacteria, and yeasts and moulds	Modified Atmosphere (70% N2/ 30% CO2)	4°C for 60 days/ 10°C for 60 days	Atas et al., 2021	
Surface inoculation						
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author	
Fresh cooked ham slices	<i>Lactococcus lactis</i> ULE383, <i>Lactiplantibacillus plantarum</i> ULE639, ULE949, ULE1599 and ULE1841, and <i>Lactiacaseibacillus paracasei</i> ULE721	<i>Listeria monocytogenes</i> CECT 911 (serovar 1/2c)	Vacuum packaging (25 mbar) Modified atmosphere packaging (80% N2/ 20% CO2) Vacuum packaging (25 mbar) + High Pressure Processing at 500MPa (15°C/ 3min) MAP trays sealed with a surface film (PET, Top Tray M/O a 47)	7°C for 19 days and 12°C for 10 days	Barcenilla et al., 2023	
Roasted pork shoulder						
Sliced emulsion type sausages	<i>Pediococcus</i> spp.	<i>Listeria monocytogenes</i>	Vacuum packaging with boards inside in vacuum bags (PA/PE) and sealed with a vacuum sealer (Allpax V 50)	7°C for 21 days	Bungenstock et al., 2020	
Cooked bacon	<i>Lactococcus lactis</i> subsp. <i>Lactis</i> (Rubis - CHR HANSEN) <i>Latilactobacillus sakei</i> (B-2 Safe Pro® - CHR HANSEN)	<i>Leuconostoc mesenteroides</i>	Packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150)	4±2°C for 90 days	Comi et al., 2016	

Table 7. List of publications on bioprotective cultures applied in seafood products, according to different strategies of incorporation.

Immersion					
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Cold-smoked salmon	<i>Latilactobacillus sakei</i> CTC 494	<i>Listeria monocytogenes</i> CTC1500	Vacuum packing in individual bags	8°C for 21 days	Aymerich et al., 2019
	<i>Latilactobacillus curvatus</i> CTC 1742	<i>Listeria monocytogenes</i> CTC1500 (<i>in vitro</i> some samples)			
Young hake Megrim	<i>Carnobacterium maltaromaticum</i> CTC1741	Total viable counts	Plastic film	0-2°C for 14 days	Gómez-Sala et al., 2016
	<i>Latilactobacillus curvatus</i> BCS35	<i>Listeria monocytogenes</i> CECT 4032			
Cold-smoked sea bass	<i>Latilactobacillus sakei</i> LAK-23	<i>Listeria monocytogenes</i>	Vacuum packed in plastic bags (PA/PE)	4°C for 60 days	Bolívar et al., 2021
	<i>Lactiplantibacillus plantarum</i> SKD4	Total viable count	Container	4°C for 5 days/ 25°C for 5 days	Jo et al., 2021
Ribbonfish	<i>Pediococcus stilesii</i> SKD11	<i>Listeria monocytogenes</i> (cepas CCUG 15527 y F11)	Vacuum packing	6 ± 2°C for 60 days	Stupar et al., 2021
	<i>Carnobacterium</i> spp.				
Cold-smoked salmon (CSS), gravlax, and sushi	<i>Leuconostoc</i> spp.	<i>Listeria innocua</i> (cepa CCUG 15531)	Vacuum packing	6 ± 2°C for 60 days	Stupar et al., 2021
	<i>Weissella</i> spp.	<i>Escherichia coli</i> (cepa CCUG 38079)			
Sprayed					
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage	Author
Fresh salmon filets	<i>Lactiplantibacillus pentosus</i> 39	<i>Listeria monocytogenes</i> ATCC 19117	Sterile plastic bag containers with normal atmospheric air	4°C for 96 hours/ 4°C for 144 hours or 4°C for 96 hours/ 30°C for 12 hours	Anacarso et al., 2014
		<i>Aeromonas hydrophila</i> ATCC 14715			
Surimi-based product	<i>Latilactobacillus sakei</i> CTC 494	<i>Listeria monocytogenes</i> CTC1034	Vacuum packed in plastic bags (PA/PE)	15 to 50 days at 2 °C and from 6 to 10 days at 12 °C	Bolívar et al., 2021
Tuna paté	<i>Latilactobacillus curvatus</i> BCS35	Total viable counts	Plastic film	0-2°C for 14 days	Gómez-Sala et al., 2016
Young hake Megrim		<i>Listeria monocytogenes</i> CECT 4032			
Col-smoked salmon	<i>Lactococcus piscium</i> EU2241	<i>Brochothrix thermosphacta</i>	Vacuum packed in plastic bags (PA/PE)	4°C for 1 week and 8°C for 3 weeks	Leroi et al., 2015
	Cold-smoked rainbow trout	<i>Leuconostoc carnosum</i> and <i>Lactococcus lactis</i>			
			<i>Listeria monocytogenes</i> CECT 4032, CECT 5366, CECT 935, CECT 5725 and CECT 7467	Vacuum packaging and refrigeration storage	Sánchez-Martín et al., 2024
Tuna flesh	<i>Lactococcus lactis</i> and <i>Levilactobacillus brevis</i>	<i>Arthrobacter</i> spp.	Sterile polythene bags	4°C and -20°C.	Sarika et al., 2016
		<i>Acinetobacter</i> spp.			
		<i>Bacillus brevis</i>			
		<i>Bacillus numilus</i>			

Table 7 (continuation)

Sprayed (continuation)				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Fish filet (Grouper family)	<i>Lactocaseibacillus rhannosus</i> GP1 and bacteriocin GP1	Coliforms <i>Aeromonas</i> spp. <i>Lactobacillus</i> spp. <i>Vibrio</i> spp.	Enfolded in sterile aluminum foil and placed in separate sterile boxes	0°C for 28 days/ 4°C for 28 days
Cooked and peeled shrimps	<i>Lactococcus piscium</i> CNCM I-4031 <i>Carnobacterium divergens</i> V41	<i>Listeria monocytogenes</i> RF191	Modified atmosphere packaging (50% O ₂ / 50% N ₂)	8°C for 28 days
Sterile syringe				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Tuna paté	<i>Latilactobacillus sakei</i> CTC 494	<i>Listeria monocytogenes</i> CTC1034	Aluminum containers with lids	15 to 50 days at 2°C and from 6 to 10 days at 12°C
Surface inoculation				
Food matrix	Protective culture	Pathogen/ spoilage microorganism	Other technologies	Storage
Surimi-based product	<i>Latilactobacillus sakei</i> CTC 494	<i>Listeria monocytogenes</i> CTC1034	Vacuum packed in plastic bags (PA/PE)	15 to 50 days at 2°C and from 6 to 10 days at 12°C
Hake fillets	<i>Lactocaseibacillus paracasei</i> spp. paracasei LAFTI-L26 and <i>Bifidobacterium animalis</i> spp. lactis LAFTI-B94	<i>Pseudomonas phosphoreum</i> CECT 4192 <i>Shewanella putrefaciens</i> CECT 5349T	Bioactive film (agar, glycerol, glucose) + vacuum packed in bags	4°C for 15 days
				López de Lacey et al., 20414

5. The protective cultures market

Clean label is no longer a fad but a lifestyle or food philosophy (Cao & Miao, 2023). It is now a requirement increasingly demanded by consumers seeking alternatives to highly processed foods. As a result, strategies that enable the food industry to obtain clean label certification are expected to grow in importance in the coming years. One such strategy is the use of protective cultures as an alternative to approved food additives.

Data regarding specific sections of food markets is publicly available in open and proprietary databases such as Market Monitor Report, Market Data Forecast, Mordor Intelligence, Market Research Engine, Research and Markets, and Data Bridge. All conclusions presented in this section are based on information gathered from these sources.

According to these insights, the market of protective cultures is expected to grow at an annual rate of 6.58% until 2029, increasing from 398.44 million USD in 2021 to 663.39 million USD in 2029.

This increase is driven by various factors that have positively influenced the commercialization of these types of ingredients. One key factor is the growing public awareness of the effects of chemical additives, which has led to an increased demand for healthier and more natural products.

Another contributing factor has been the occurrence of the Covid-19 pandemic, which has not only had an economic impact on the food industry and associated industries but also heightened consumers and legislative awareness of the importance of clean label products. Due to this behaviour change and the varying consequences across countries, the protective cultures market is adopting different strategies for each of them. The increased consumption of dairy products and the use of protective cultures for the

preservation of soured foods, including dairy products (yogurts, cheeses, sour cream) and meat products (sour sausages), also contributed to this trend.

Furthermore, improvements and advances in technology, as well as distribution channels, have facilitated the commercialization of these ingredients. Finally, the strict and variable standards for ensuring food security play a crucial role in driving the market for protective cultures.

5.1. The role of producing countries in the protective cultures market

Europe leads the global market for protective cultures and bacteriocins, holding a 44% share according to market studies conducted between 2022 and 2029 by Mordor Intelligence (2023-2028), Maximize Market Research (2022-2029), Market Data Forecast (2023-2028) and Data Bridge Market Research (2022-2029). This dominance could be attributed to the region's historical significance in the dairy sector, evolving consumer concerns, increased production and consumption of dairy products, and advancements in dairy food production. Additionally, the use of protective cultures and bacteriocins as additives in dairy, meat, and seafood products has risen, driven by the need for longer shelf-life products to address food corruption concerns.

In the case of Asia-Pacific region, market growth is anticipated due to a shift towards a more Westernized lifestyle. This includes increased consumption of organic and natural (clean label) products and dairy products, as well as heightened awareness and knowledge about protective cultures in countries such as India and China.

Finally, in North America, market growth is partly driven by increased consumption of dairy, meat, and seafood products, like trends observed in the Asia-Pacific region. Additionally, consumer concerns for safe and ready-to-eat (RTE) foods, facilitated using natural food preservatives, are propelling the market for protective cultures in this region.

5.2. The world's largest producers of protective cultures and bacteriocins

The top 10 list of the most important international commercial producers of protective cultures products consists of 70% of European companies, highlighting the value of this alternative in the European Union. In general, the market is currently highly fragmented, as illustrated in the accompanying graph taken from Mordor Intelligence. There are two types of players: international players, who occupy a smaller share, and national players, who compete intensely within their regions. Consequently, the market is highly competitive with no dominant operators.

The most frequently mentioned producers in market studies by Mordor Intelligence (2023-2028), Maximize Market Research (2022-2029), Market Data Forecast (2023-2028), and Data Bridge Market Research (2022-2029) are Chr. Hansen, THT, Bioprox, and DSM. Each of these companies offers a variety of starter and protective cultures tailored for different processes and food products.

5.2.1. Chr. Hansen (<https://www.chr-hansen.com>)

The company is a leading provider of bacterial cultures for food applications, recognized as one of the most esteemed culture companies in Europe. Its product portfolio includes enzymes, probiotics, and starter cultures, which facilitate the production of fermented products and the achievement of specific organoleptic properties. Additionally, the company offers protective cultures for both fermented and non-fermented foods, marketed as clean label ingredients and categorized based on their intended food applications. Currently, there are five primary alternatives. A notable product is FRESHQ[®], designed for fresh dairy products such as yogurt, sour cream, quark, kefir, tvorog, white cheese, cottage cheese, and pasta filata, which received the World Dairy Innovation Awards 2021. For cheese, the BIOSAFE[®] culture is utilized, while SAFEPRO[®] is tailored for meat and processed meat products. The VEGA[™] portfolio is

applied in fermented vegetables, with VEGA™ SAFEPRO® specifically for meat analogues. Lastly, VINIFLORA® is dedicated to wine production.

5.2.2. THT S.A. (<https://www.tht.be/>)

They are specialists in meat starter probiotics for products such as meat, fermented meat, and sausages. They offer various strains with different effects on the products. Strains aimed at product acidification include *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus*. Strains oriented towards enhancing organoleptic or sensory characteristics are *Staphylococcus carnosus* and *Staphylococcus xylosus*. Additionally, they provide health-related active strains.

5.2.3. BIOPROX pure cultures (<https://www.bioprox.com>)

The company offers an extensive portfolio of starter and protectant cultures, primarily targeting dairy products. These offerings are categorized based on the type of product to which the protective culture is applied. Dairy products constitute the core of the portfolio, featuring clean label alternatives for butter, such as the LACTO-PROX and MEDIA-PROX ranges. The YO-PROX range includes starter cultures for set yogurts, stirred yogurts, and drinkable yogurts. Additionally, DI-PROX and AROMA-PROX provide specialized starters for fermented milk-derived cheeses. For other fermented dairy products, DI-PROX®K and DI-PROX MTTX® are available for kefir and dahi, respectively. The BIOPROX range comprises bioprotective cultures designed for fermented milk yogurt and cheese technologies, effectively reducing and inhibiting yeasts, moulds, and spoilage microorganisms in dairy products and cheeses. In response to the growing demand for plant-based products, the company also offers a vegan range under the VEGI-PROX range.

5.2.4. DSM-Firmenich (<https://www.dsm-firmenich.com/>)

The company offers a range of protective cultures, primarily aimed at extending the shelf life of cheeses. Delvo®Cid is a biopreservative based on the bacteriocin natamycin, suitable for protecting various products such as bread, buns, cakes, and dairy products. Delvo®Guard and Dairy Safe™ are designed to prevent the presence of moulds and yeasts in cheeses, while also acidifying and providing desired organoleptic properties. Additionally, Delvo®Zyme is used to prevent the growth of *Clostridium* in cheese products.

6. Conclusions

Bioprotective cultures are a sustainable and effective clean label alternative for food preservation, aligning with consumer expectations for natural and minimally processed foods. Their ability to inhibit spoilage and pathogenic microorganisms without negatively affecting sensory properties makes them a valuable tool for the food industry. The scientific evidence and market trends confirm their increasing relevance across diverse food sectors, including fruits, vegetables, dairy, cereals, meat, and seafood. However, challenges remain regarding regulatory approvals, consumer acceptance, and ensuring consistent efficacy across food matrices. Continued research, including well-designed industrial trials, is essential to optimize the application of bioprotective cultures, sustain the basis for the clarification of legal frameworks, and evaluate their effectiveness under real processing conditions. Overall, bioprotective cultures represent a key strategy in advancing towards sustainable food preservation while meeting the demands of the clean label market.

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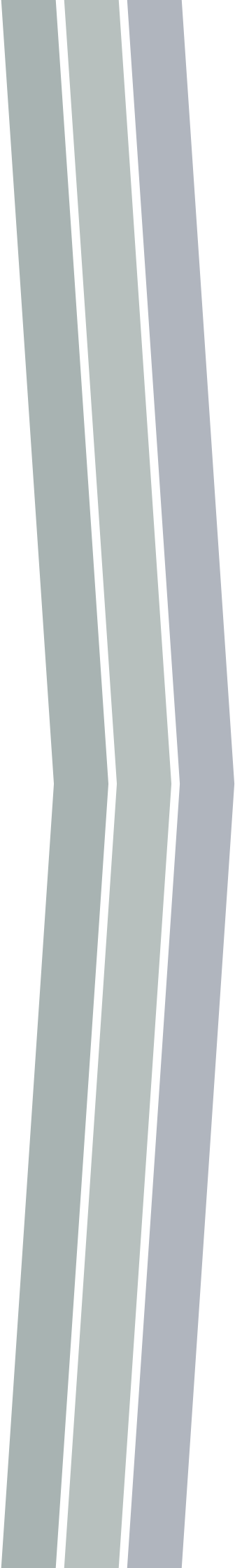
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JUSTIFICACIÓN y OBJETIVOS



Justificación y objetivos

La tendencia *clean label* (etiqueta limpia) en la industria alimentaria hace referencia al desarrollo de productos con listados de ingredientes simplificados y fácilmente reconocibles por el consumidor, que busca evitar o minimizar el uso de aditivos de la lista positiva europea, identificados por números E, especialmente aquellos considerados artificiales. Esta estrategia responde a la evolución del mercado y la creciente demanda de transparencia, naturalidad y percepción de seguridad en los alimentos, y suele complementarse con un etiquetado claro y comprensible.

Con el fin de ofrecer al consumidor productos *clean label* que sean sanitariamente seguros y cuyas propiedades organolépticas no cambien sustancialmente con respecto al producto fresco, es crucial el diseño de estrategias de conservación sostenibles que sirvan como alternativas al uso de tecnologías físicas y conservantes de la lista positiva (números E-).

En el contexto actual, caracterizado por una creciente demanda de alimentos más naturales y con etiquetas limpias, una de las estrategias emergentes que puede ayudar a la industria alimentaria a dar respuesta a estas expectativas es el biocontrol, entendido como el empleo de cultivos bacterianos, conocidos como cultivos protectores, con actividad antimicrobiana, bien como coadyuvantes tecnológicos durante el proceso de elaboración, bien como ingredientes incorporados en la formulación del alimento.

La estrategia de biocontrol ha experimentado una evolución notable en los últimos años. Inicialmente centrada en el uso de cultivos iniciadores con capacidad antimicrobiana en alimentos fermentados, ha dado paso a nuevas aplicaciones, como el empleo de mezclas de cultivos bacterianos con otros ingredientes en formato polvo, comercializados por su potencial conservante y de aplicación en alimentos no fermentados.

Estos cultivos protectores ejercen su efecto conservante gracias a la producción de compuestos antimicrobianos tales como ácidos orgánicos, peróxido de hidrógeno y, especialmente, péptidos antimicrobianos de bajo peso molecular denominados bacteriocinas. Estas últimas han sido ampliamente estudiadas por su capacidad para inhibir microorganismos sin acidificar la matriz alimentaria, lo que permite preservar las propiedades organolépticas del producto final. No obstante, su aplicación industrial está limitada, ya que la legislación alimentaria europea solo autoriza el uso de la bacteriocina nisina, producida por *Lactococcus lactis*, como aditivo conservador.

Las restricciones normativas sobre el uso de otras bacteriocinas han impulsado la exploración de alternativas como el empleo del cultivo bacteriano completo o, incluso, del sobrenadante libre de células (SLC), obtenido tras la eliminación de la biomasa microbiana, y que también puede contener compuestos con actividad antimicrobiana.

En este marco, el objetivo general de la presente tesis doctoral es **definir estrategias de conservación de alimentos alineadas con el enfoque *clean label*, basadas en el uso de cultivos protectores o sus SLC, con actividad frente a microorganismos alterantes presentes en alimentos *ready-to-eat*, con el fin de prolongar su vida útil y minimizar, o incluso eliminar, el uso de aditivos autorizados en la lista positiva europea.**

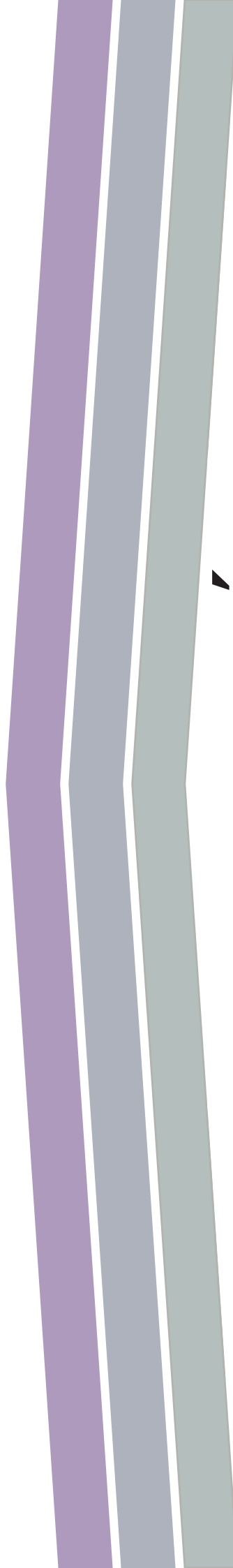
Las estrategias desarrolladas deberán ser transferibles a escala industrial, incluyendo la definición del procedimiento y formato de aplicación (en proceso o producto), así como aspectos clave como el etiquetado y la evaluación sensorial del alimento final.

Para alcanzar este objetivo, se ha trabajado con una colección propia de más de 500 cepas bacterianas con potencial biotecnológico del Centro Nacional de Tecnología y Seguridad Alimentaria (CNTA).

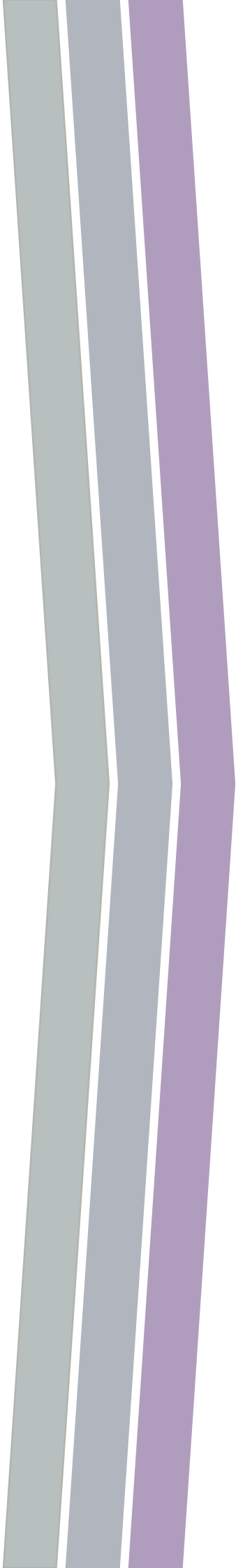
Para lograr eficazmente este objetivo general, se establecieron los siguientes objetivos técnicos específicos:

1. Caracterización y selección de las cepas bacterianas candidatas a cultivos protectores a partir de la colección de CNTA:
 - 1.1. Caracterización y cuantificación de la actividad antimicrobiana de los SLC de los potenciales cultivos protectores frente a microorganismos patógenos y alterantes de interés en alimentos, y la posterior selección de las cepas con mayor actividad conservante.
 - 1.2. Caracterización de la naturaleza de los compuestos responsables de la actividad antimicrobiana (ácidos orgánicos, peróxido de hidrógeno, bacteriocinas, etc.) de los SLC de las cepas candidatas.
 - 1.3. Caracterización de la seguridad sanitaria de las cepas seleccionadas incluyendo la determinación de la resistencia a antibióticos y de su potencial toxigénico.
 - 1.4. Determinación del perfil genómico de las cepas seleccionadas mediante el análisis bioinformático.
 - 1.5. Determinación de la estabilidad térmica de los SLC bajo condiciones reales de tratamientos de la industria alimentaria.
2. Desarrollo, en colaboración con empresas de la industria alimentaria, de estrategias *clean label* para extender la vida útil y eliminar el uso de aditivos con número E:
 - 2.1. Aplicación de los SLC en sticks de piña para inhibir el crecimiento de los microorganismos responsables del fin de vida útil.
 - 2.2. Aplicación de los SLC en bocados *plant-based* tipo “pollo” para inhibir el crecimiento de microorganismos responsables de la alteración del producto.

- 2.3. Aplicación de los SLC en gazpacho fresco para inhibir el crecimiento de los microorganismos responsables de alterar el producto (los resultados relativos a este objetivo se describen brevemente en el apartado de la discusión).
3. Análisis de las limitaciones y oportunidades del marco regulatorio europeo en relación con el etiquetado de los cultivos protectores y sus derivados, con el objetivo de identificar vías legales viables para su incorporación como ingredientes en alimentos no fermentados, garantizando la transparencia y el cumplimiento normativo.



MATERIAL y MÉTODOS



Material y Métodos

Debido a que en cada una de las publicaciones incluidas en esta tesis doctoral se describe la metodología en detalle en el apartado de “material y métodos”, en esta sección únicamente un capítulo de un libro resume de manera esquemática y simplificada los principales procedimientos para alcanzar el Objetivo 1; relacionado con el Manuscrito II y Manuscrito III.

Asimismo, se incorpora una descripción esquemática del desarrollo de las tres estrategias de conservación *clean label*, realizados en colaboración con empresas del sector agroalimentario, en el marco del Objetivo 2. De los nuevos productos desarrollados, los sticks de piña y los bocados *plant-based* serán descritos en manuscritos científicos,

- a) Manuscrito V: Quality and safety preservation of pineapple sticks using cell-free supernatant of protective cultures
- b) Manuscrito VI: Extending the shelf life of plant-based meat analogues by adding cell-free supernatant from *Lactiplantibacillus plantarum*

En el caso del tercer producto, el gazpacho, estará descrito en la discusión final de esta tesis industrial.

Manuscrito II

Selection and genotypic characterization of potential bacterial strains for use as protective cultures

Nerea Garín-Murguialday, Raquel Virto, Rafael Pagán and Laura Espina*

Chapter x

Selection and genotypic characterization of potential bacterial strains for use as protective cultures

Nerea Garín-Murguialday, Raquel Virto, Rafael Pagán, Laura Espina

Abstract

Antimicrobial compounds produced by bacteria, such as lactic acid bacteria and *Bacillus* spp., are alternatives to artificial preservatives, enabling companies to meet customer demand for clean-label products. To comply with the relevant EFSA's specifications, it is important to genotypically and phenotypically characterize the cultures that produce antimicrobial compounds. This chapter summarizes the steps necessary to characterize the strains originating the protective cultures for their potential use as ingredients in the food industry.

Keywords Protective cultures, Cell-free supernatant, Antimicrobial activity, Bacteriocins, Antibiotic resistance, Whole genome sequencing.

1 Introduction

Food on the market must be safe and innocuous, so preservation is a fundamental step to producing products with an adequate shelf life, suitable for consumption. Preservation must cope with two aspects. On the one hand, it should be controlling pathogenic microorganisms such as *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*, which cause foodborne diseases (1); and on the other hand, it should reduce food waste often caused by bacteria, moulds (*Penicillium* spp., *Aspergillus* spp.) and yeasts (2, 3).

Companies are continuously working to develop a variety of techniques to inhibit microbial growth. Conventional technologies such as heat treatments (pasteurization and sterilization), cooling techniques (refrigeration and freezing), reduction in water activity (spray-drying and freeze-drying), fermentation, and chemical preservatives (4) have been instrumental in maintaining food safety. However, these processes do not fully align with consumers' desire for products that retain the organoleptic and nutritional characteristics of fresh products (5). Consequently, the industry faces the dual challenge of extending product shelf life while aligning strategies with current consumer trends, such as the clean label movement (6).

sumers' demand, various novel preservation strategies have emerged, including the use of bioprotective bacterial cultures and their metabolites (7). This approach involves utilizing microorganisms or their products, such as cell-free supernatants (CFSs), that act through competitive exclusion or the production of antimicrobial substances (8). Following European Commission's requirements on the characterization of production microorganisms used in feed (9, 10), the selected microbial species must belong to the Qualified Presumption of Safety (QPS) (11) and must not possess any acquired transferable antibiotic resistance genes to prevent horizontal gene transfer. Furthermore, there must be evidence of the non-toxicogenicity of the cultures, the stability of the intended technological effect, and the definition of safe usage levels (12, 13). Among the possible options, lactic acid bacteria (LAB) stand out due to their well-established tradition in food applications, their perception as safe for human consumption, and the exhibition of a broad spectrum of antimicrobial activity (AM) (12). Another interesting potential protective culture is based on the exploitation of some *Bacillus* spp., which produce a wide range of antimicrobial and antifungal compounds (14).

In this chapter we describe a methodology for the comprehensive evaluation of potential protective cultures and the verification of their compliance with all the relevant EFSA's specifications, combining phenotypic assays and genome analysis of sequencing data. More specifically, we summarize how to measure the antimicrobial activity by agar well diffusion assay and by minimum inhibitory concentration (MIC) determination, assess antibiotic resistance and cytotoxicity, infer the nature of the antimicrobial compounds and their thermal stability, and analyse genomic data for taxonomic confirmation and identification of antibiotic resistance genes (ARGs) and biosynthetic gene clusters (BGCs) involved in secondary metabolite production. We have validated this protocol for lactic acid bacteria and *Bacillus subtilis*, but it can be applied for any potential protective culture. In addition, we offer tips to show you how to prepare the material, follow the methods, and interpret the results.

2 Materials

2.1 Culture preparation

1. Cryovial of the indicator microorganisms
2. Cryovial of the potential protective culture microorganism
3. Broth selected for optimal growth of each microorganism
4. Sterile bottles of 10 and 50 mL
5. Agar selected for optimal growth of each microorganism

6. Sterile petri dishes (90 mm)
7. Micropipettes
8. Sterile pipette tips
9. Microtubes (1.5 mL)
10. Buffered peptone water (0.1 %)
11. Incubator
12. Centrifuge
13. Single-use 500-mL vacuum filter unit with 0.22 μ m PES filter
14. Sterile plastic bottles

2.2 Measurement of antimicrobial activity of the CFS using the agar well diffusion method

1. Sterile petri dishes (90 mm)
2. Optimum growth agar of the indicator microorganism
3. Sterile borer (7.20 mm)
4. Calliper or ruler
5. Buffered peptone water (0.1 %)
6. Digrafsky seeding loops
7. Sterile petri dishes (90 mm)
8. Optical microscope
9. Incubator

2.3 Determination of the MIC of the CFS using the broth microdilution assay

1. 96-well flat-bottom microtiter plate
2. Micropipettes
3. Sterile pipette tips
4. Microtubes (1.5 mL)
5. Optimum growth broth of the indicator microorganism
6. Sterile bottles of 10 and 50 mL
7. Spectrophotometer
8. Incubator

2.4 Measurement of the antibiotic resistance of the protective cultures using the broth microdilution assay

1. Sterile petri dishes (90 mm)
2. Anaerobiosis cabinet
3. Bunsen burner
4. 96 - well flat - bottom microtiter plate
5. Multichannel pipette
6. Micropipetter and sterile pipette tips
7. Pipettor and sterile serological pipettes (10 and 25 mL)
8. Sterile microtubes (1.5 and 2 mL)
9. Sterile filters (0.22 μ m)
10. Sterile falcon (15 and 20 mL)
11. Antibiotics (kanamycin, gentamicin, streptomycin, tetracycline, clindamycin, chloramphenicol, ampicillin, erythromycin, and vancomycin)

12. Incubators
13. Iso-Sensitest (IST) broth
14. Man Rogose y Sharpe Medium (MRS broth and agar)
15. Screw-capped glass culture tubes
16. UV/ VIS spectrophotometer
17. Spectrophotometer cuvettes
18. Anaerobiosis jar
19. Anaerocult® for microbiology
20. Sterile NaCl 0.85%
21. MiliQ sterile water

2.5 Measurement of cytotoxicity of the CFS using the MTT assay

1. Caco-2 cell line
2. HT-29 cell line
3. 3-(4, 5 - dimethylthiazol - 2 - yl) - 2, 5 - diphenyltetrazolium bromide
4. 96-well flat- bottom microtiter plate
5. Micropipettes
6. Sterile pipette tips
7. Incubator CO₂ control
8. Freezer (-80°C)
9. Phenol red-free medium
10. Dimethyl sulfoxide (DMSO)
11. Spectrophotometer

2.6 Determination of the nature of the antimicrobial of the CFS

1. NaOH 1 M
2. HCl 1 M
3. Proteinase K
4. Phosphate
5. Pepsin
6. Sterile pasteur pipettes
7. Incubator
8. Micropipettes
9. Sterile pipette tips
10. Optimum growth agar of the indicator microorganisms
11. Digralsky seeding loops
12. L-shaped spreaders
13. Sterile petri dishes (90 mm)
14. Sterile borer (7.20 mm)
15. Caliper or ruler
16. Buffered peptone water (0.1 %)
17. Microtubes (1.5 mL)

2.7 Determination of the thermal stability CFS

1. Sterile micropipettes
2. Sterile pipette tips
3. Vials (2 mL)
4. Microtubes (2 mL)
5. Box with cold water or ice
6. Thermoblock
7. Calibration oil bath
8. Optimum growth agar of the indicator microorganisms
9. Digrafsky seeding loops
10. L-shaped spreaders
11. Sterile petri dishes (9 mm)

2.8 DNA extraction, whole genome sequencing (WGS) and post-sequencing analysis of each potential culture

2.8.1 DNA extraction

1. Pure culture of the protective strain
2. DNA extraction kit (GenElute Bacterial Genomic DNA kit, NA2110, Sigma – Aldrich, Missouri, USA)
3. Microcentrifuge
4. Micropipettes
5. Microtubes
6. Pipette tips

2.8.2 WGS

Illumina-based whole genome sequencing service

2.8.3 Post-sequencing analysis

1. Paired-end raw FASTQ files with short reads generated from the submitted DNA
2. Bioinformatic tools and their dependencies accessed on a local Linux server or via their standalone GUI application, or Alternatively using the European Galaxy Server (15):
 - a) Trimmomatic v0.39 (16)
 - b) FastQC v0.12.1 (17)
 - c) Kraken2 v2.1.3 against a comprehensive and updated database (18)
 - d) Shovill v1.1.0 (19)
 - e) QUAST v5.2.0 (20)
 - f) ABRicate v1.0.1 (21) with CARD (22, 23) and Resfinder (24, 25) databases
 - g) antiSMASH v6.1.1 (26)
 - h) BAGEL4 (27)

3 Methods

3.1 DNA extraction

The first step in the analysis is the preparation of the cultures of the indicator microorganisms (28-30) and the potential protective cultures. The selection of the indicator microorganisms, whether pathogenic or spoilage, should be guided by their associated effects and prevalence in the targeted food products. Some potential indicators include *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. [1]. For both indicator microorganisms and protective cultures, it is essential to conduct a preliminary study to determine the optimal growth conditions for each bacterial strain, including the choice of broth and agar media, incubation temperature, atmospheric conditions, and growth time.

3.1.1 Preparation of the cultures of the indicator microorganisms

Inoculate and spread the indicator strain from the cryovial on the surface of agar plates with an inoculation loop to isolate colonies and then incubate the plates under the indicator microorganism's optimal growth conditions. Select 2 colonies of the plate, inoculate them into 10 mL of broth in a glass bottle with caps, and incubate the inoculated bottles under the optimal growth conditions. After that time, inoculate 50 mL of growth broth in a glass bottle with 100 μ L of the previous culture and incubate the inoculated bottle (see Note 1).

3.1.2 Preparation of the cultures and collection of the cell-free supernatants (CFS)

Figure 1 shows how to prepare the protective culture's CFS. Select a potential protective culture strain from the QPS list drawn up by EFSA and spread it on the surface of agar plates selected for their optimal growth with an inoculation loop. Incubate the plate under the protective microorganism's optimal growth conditions. Inoculate 3 - 4 colonies of it into 50 mL of growth broth in a glass bottle with caps and incubate them. Centrifuge 50 mL of the culture at $4347 \times g$ for 10 min at 4°C to obtain the supernatant. Filter it with a $0.22 \mu\text{m}$ filter and store the CFSs at 4°C .

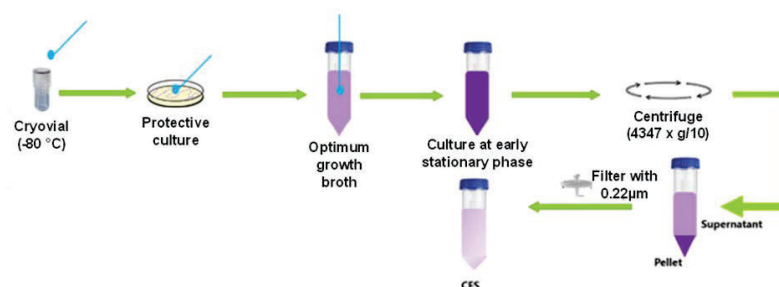


Fig. 1 Methodology to obtain the CFS of protective cultures.

3.2 Measurement of antimicrobial activity of the CFS using the agar well diffusion method

Pour 20 mL of the agar selected for the optimal growth of the indicator strain and let it solidify. Then, add and spread 10⁶ CFU/mL of the indicator strain with a Digralsky loop and punch a well of 7.2 mm in diameter in each plate with the agar using a sterile borer. Fill each well with 100 μ L of the testing CFS. Finally, keep the plates for 2 h on the bench and then incubate them at the optimal growth conditions of the indicator strain as indicated in Figure 2.

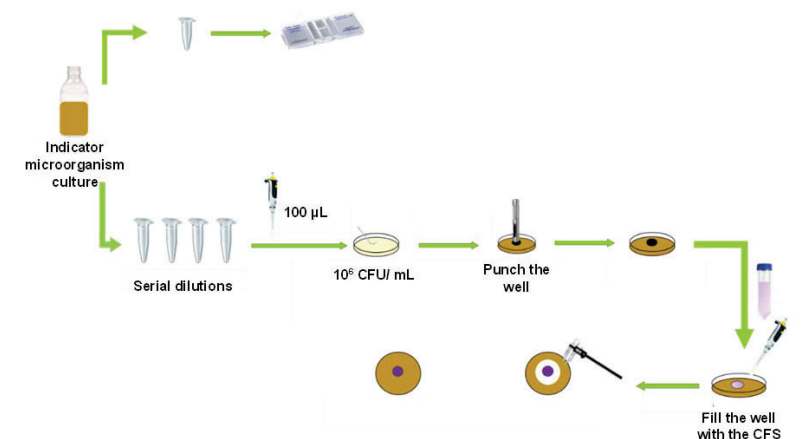


Fig. 2 Agar well diffusion method.

Measure the halo the way Figure 3 shows and select the strains with the highest antimicrobial activity (see Note 2). Table 1 represents the correlation between the halo measurement and the inhibition zone.

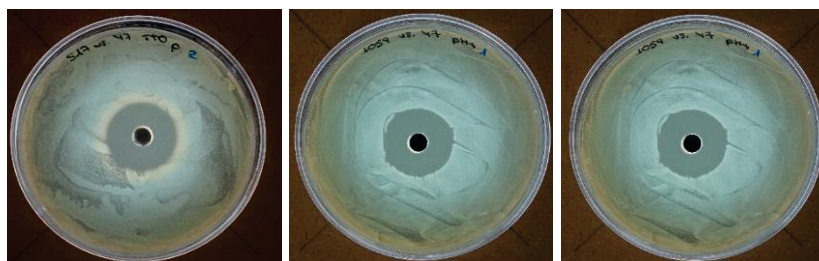


Fig. 3 Petri dishes showing bacterial growth inhibitory by agar diffusion.

3.3 Determination of the MIC of the CFS using the broth microdilution assay

Add to each well of a sterile 96-well flat-bottom microtiter plate 180 μ L of the selected broth for the optimal growth broth of the indicator strain. Fill the wells to reach variable concentrations of the CFSs (0%, 0.15%, 0.31%, 0.625%, 1.25%, 2.5% and 5%) and with the indicator microorganism at a final concentration of 10³ CFU/mL and 10⁵ CFU/mL. Incubate the plates under optimum growth conditions for the indicator microorganism and measure the optical density at a wavelength of 620nm at different times (see

Note 3). With the obtained results determine the MIC as the lowest concentration of a compound necessary to inhibit bacterial growth.

3.4 Measurement of the antibiotic resistance of the protective cultures via the broth microdilution assay

The determination of antibiotic resistance of the strains is carried out phenotypically using the modified ISO 10932/IDF 223 standard (31). First, seed the bacteria to be tested and incubate them in their optimal growth conditions by streaking by depletion and in parallel prepare the 0.85% NaCl, MRS Broth, IST Broth and miliQ water solutions and the antibiotic stocks and store them at - 20 °C. Add the culture medium to the wells of the microtiter plate and 10 µL of each of the antibiotics in column 2 and in the rows indicated in the ISO. Resuspend in 5 mL of the saline solution (NaCl 0.85%) the amount of colonies of the bacteria to be tested to an OD nm of 0.16 - 0.2 ($3 \cdot 10^8$ CFU/mL). Dilute the above suspensions at 1/500 in the appropriate culture medium and distribute 50 µL of the 1/500 diluted bacterial suspension from column 2 to column 12 per well. To finish, incubate at the appropriate temperature and conditions for 48 h.

3.5 Measurement of cytotoxicity of the CFS using the MTT assay

The in vitro assessments are conducted using Caco-2 and HT-29 cell lines, both human colorectal cancer cells, by MTT method (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) to determine if the strains to be used as ingredients prove cytotoxic to human health (32). Seed the cell lines in a 96- well flat-bottom microtiter plate ($5 \cdot 10^3$ cells/well for the Caco-2 and $1.7 \cdot 10^4$ cells/well for the HT-29 cell line) and then incubate them at 37 °C (48 h, 5% CO₂, and 95% relative humidity). For the HT-29 cell line, directly add the CFS at different concentrations. For the Caco-2 cell lines, refresh the medium three times per week and after 15 days introduce the CFS at varying concentrations. Transfer the entire volume to 96-well-U-bottom microtiter plates and store at - 80 °C. Afterwards, remove the culture medium and add to each well 200 µL of phenol red-free medium and 50 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) at a concentration of 5 mg/mL. Incubate the plates at 37 °C (3 h, 5% CO₂, and 95% relative humidity) and eliminate the unmetabolized MTT. When eliminated, add 150 µL/well of dimethyl sulfoxide (DMSO) and agitate at 347.76 × g for 5 min. Transfer 100 µL of the supernatant to a new 96-well flat-bottom plate to measure the optical density at a wavelength of 570 nm. Finally, calculate the viability of the cell lines using Equation 1.

$$\text{Eq. 1} \quad \% \text{ viability} = \frac{OD_{570} \text{ sample}}{OD_{570} \text{ control}} \times 100$$

3.6 Determination of the nature of the antimicrobial compounds of the CFS

To ensure that the antimicrobial activity of CFS is due to the production of bacteriocins by the strain, neutralization (1.a), acidification (1.b) or addition of proteases (1.c – 1.d) to the CFS is carried out.

1.a. Add 1 M NaOH to the CFS. (pH 7.00) (33, 34).

1.b. Add 1 M HCl to the CFS. (pH 3.50) (33, 34).

1.c. 25 µL of proteinase K to 5 mL of CFS (35).

Perform measurement of antimicrobial activity of the CFS using the agar well diffusion method (3.2) and measure the halos and determine whether the activity is due to the organic acids or to the production of bacteriocins (see Note 4).

3.7 Determination of the thermal stability of the CFS

The heat resistance of the CFSs is evaluated to determine whether their antimicrobial activity would remain effective when incorporated into foods prior to pasteurization or sterilization treatments commonly used in the food industry. These treatments were used as a reference to select the intensity (temperature and time) of the different heat treatments (from 10 to 120 min at 70, 90, 110, and 120 °C).

To perform it, add a small volume (1 mL) of the CFS to a vial with 9 mL of preheated treatment medium (f.i. culture media, buffer, liquid food, etc.) and introduce it into a preheated liquid at the selected temperature (see Note 5).

After the established time remove the vials and place them in cold water for thermal shock. Repeat the measurement of the antimicrobial activity of the CFS using the agar well diffusion method (3.2) with the treated CFS. Measure the halo obtained and compare it with the original halo made with the CFS without treatments (see Note 6) using Equation 2.

Eq. 2 % loss antimicrobial activity =

$$\frac{\text{halo diameter (mm)before heat treatment} - \text{halo diameter (mm)after heat treatment}}{\text{halo diameter (mm)before treatment}} \times 100$$

3.8 DNA extraction, WGS and post-sequencing analysis of each potential culture for the determination of ARGs and BGCs

Besides carrying out experimental procedures for the determination of the antibiotic resistance and of the potential nature of the antimicrobial compounds, WGS and post-sequencing analysis should also be carried out to help reveal the possible ARGs and the potential BGCs involved in secondary metabolite production, respectively.

3.8.1 DNA extraction

A pure culture of the potential protective strain must undergo DNA extraction with an adequate DNA extraction kit, following its instructions.

3.8.2 WGS

A sequencing provider can perform DNA quality checking, library preparation and sequencing on an Illumina platform, delivering high-quality sequencing reads with Phred scores above 30 for most bases.

3.8.3 Post-sequencing analysis

The resulting paired-end raw FASTQ files are then processed following the steps showed in Figure 4. First, pre-process the 2×150 -bp paired-end sequencing reads generated by removing adapters and short reads using Trimmomatic v0.39 with default settings for sliding window quality control, leading/trailing base quality trimming, and a minimum length of 36 bp and check the quality of the filtered clean reads with FastQC v0.12.1. Then use Kraken2 on the raw FASTQ reads to classify sequences against a comprehensive database (e.g., bacterial, archaeal, viral) to confirm the taxonomic identity of the bacterial isolate and the absence of contaminants and perform the novo genome assembly with Shovill v.1.1.0 using the SPAdes assembler with default parameters. Evaluate the quality of the assembly with QUAST v5.2.0. and, as an optional step, run Kraken2 again on the FASTA file for additional confirmation of the taxonomic identity of the assembled contigs. Screen the assembled contigs in FASTA format for ARGs using the ABRicate v1.0.1 tool with the CARD and ResFinder databases with a minimum identify and coverage values of 80%. Finally, predict the type and location of BGCs involved in secondary metabolite biosynthesis within the assembled genomes with antiSMASH v6.1.1 (see Note 7).

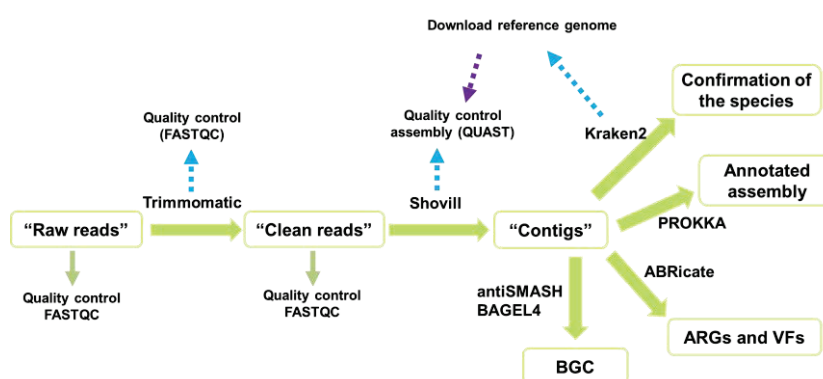


Fig. 4 Bioinformatic analysis of the WGS of the antimicrobial strain.

4 Notes

1. To verify the efficacy of the filtration process, streak the obtained CFS and incubate it under the growth conditions of the protective bacteria. If the filtration is inadequate, repeat the filtration process.
2. When quantifying the halos, it must be considered that not all halos are positive, but only those exceeding the diameter of the well (7.2 mm) by 2 mm (> 9.2 mm).
3. The MIC can be determined in two ways: by measuring at different intervals after removing the plate from the incubator, or by using an incubator that continuously measures while maintaining incubation conditions, thereby obtaining the growth curves of the indicator microorganisms in the presence of various concentrations of the CFS.
4. If the halo disappears when neutralizing the CFS, the antimicrobial activity can be attributed to the organic acids produced by the bacteria. If the halo disappears after the addition of the protease, the antimicrobial activity of the CFS can be ascribed to the production of proteinaceous bacteriocin or bacteriocin-like substances. However, if the halo persisted, other proteases such as pancreatin should be added to the tests.
5. To ensure the treatment starts at the specified time, the temperature of a separate vial containing CFS under identical conditions must be monitored with a temperature probe.
6. With these results make a linear regression to estimate the loss of antimicrobial activity of the CFS at different commercial heat treatments.
7. The tool BAGEL4 is also recommended for this purpose.

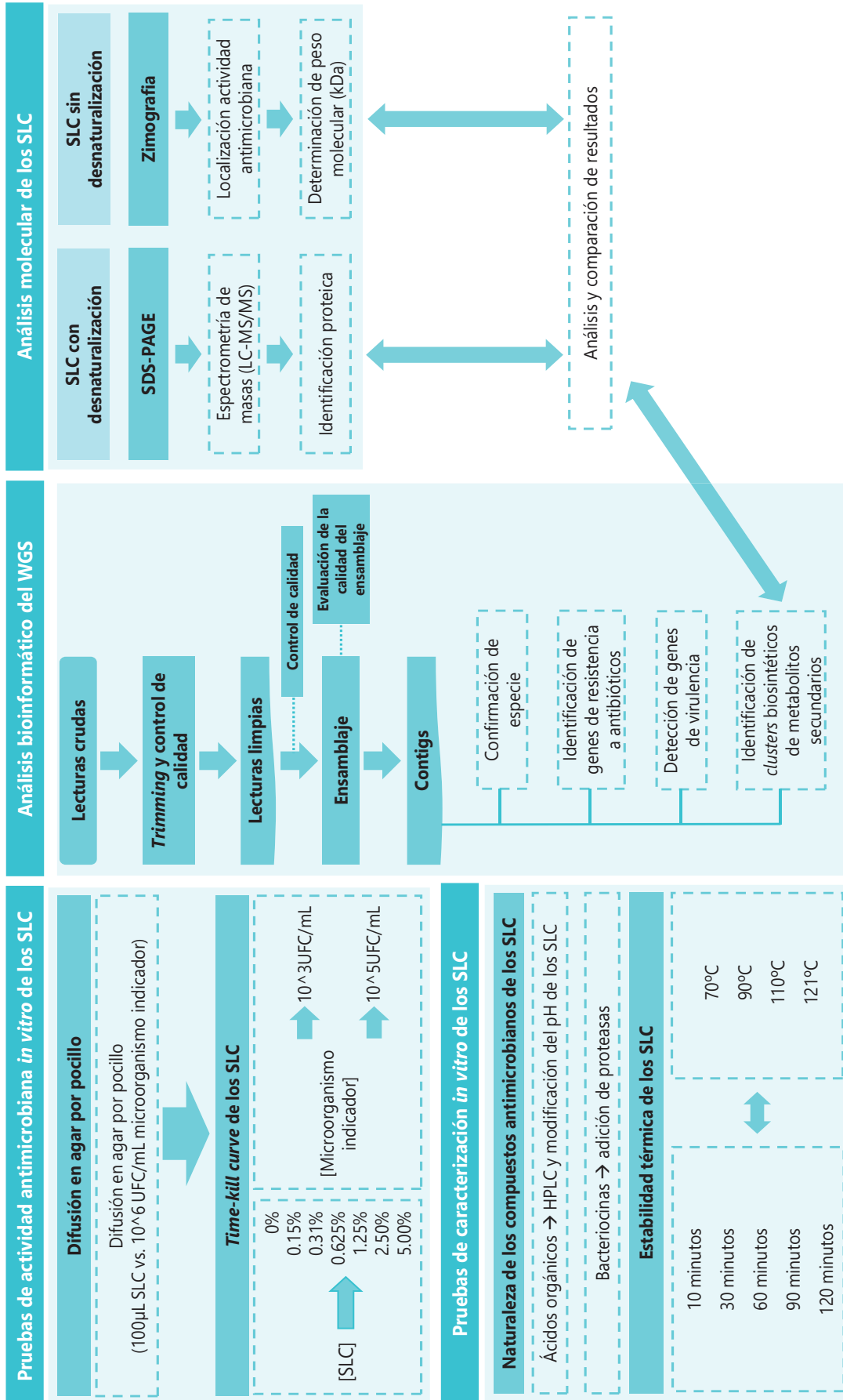
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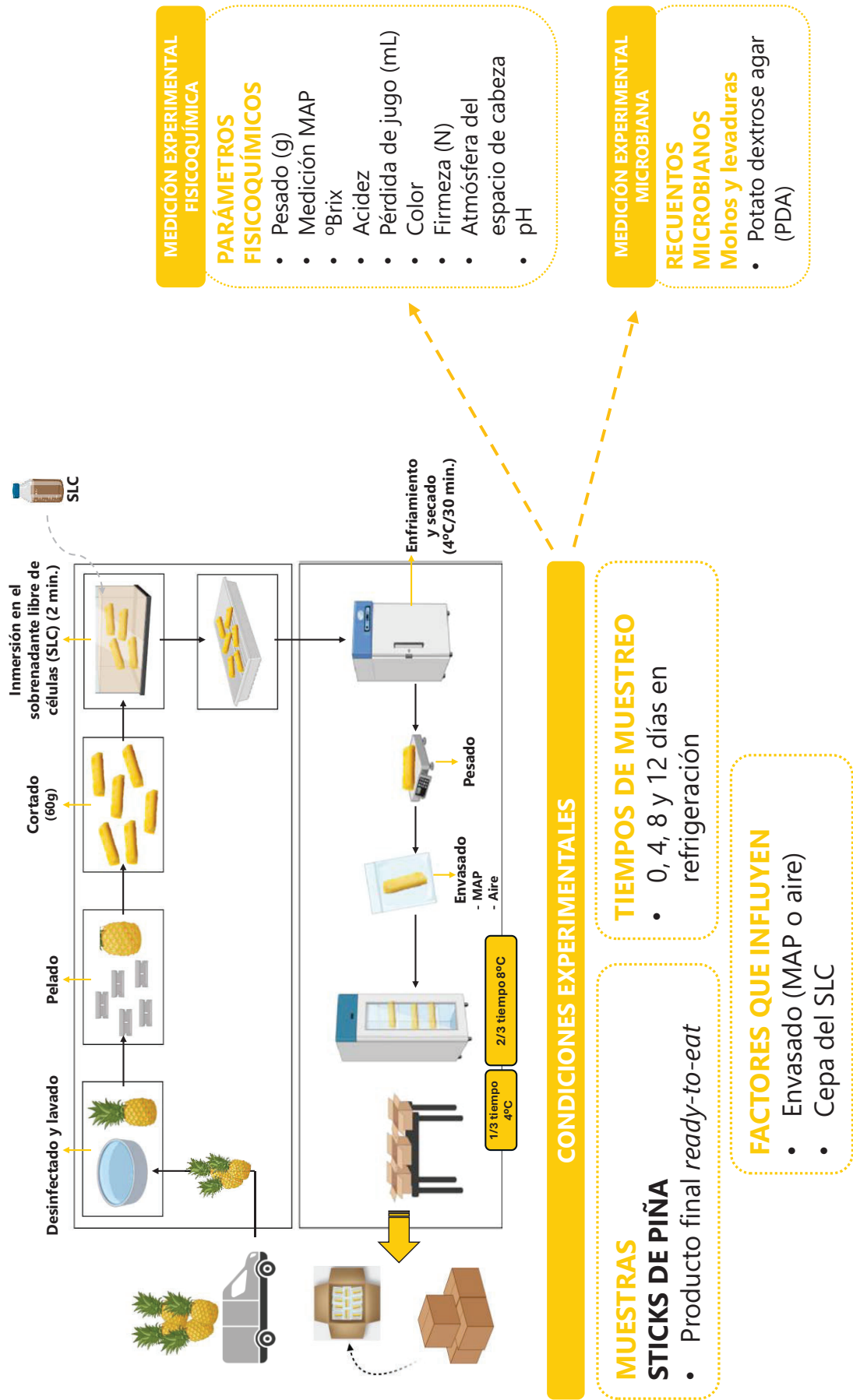
This research was supported by the University, Innovation and Digital Transformation Department, R&D&I Service of the Government of Navarra (Spain) with the funding allocation G20001-G2100-4701- 467300: Contratación personal investigador y tecnológico, doctorados industriales—project BacBiop (0011-1408-2022-000003).

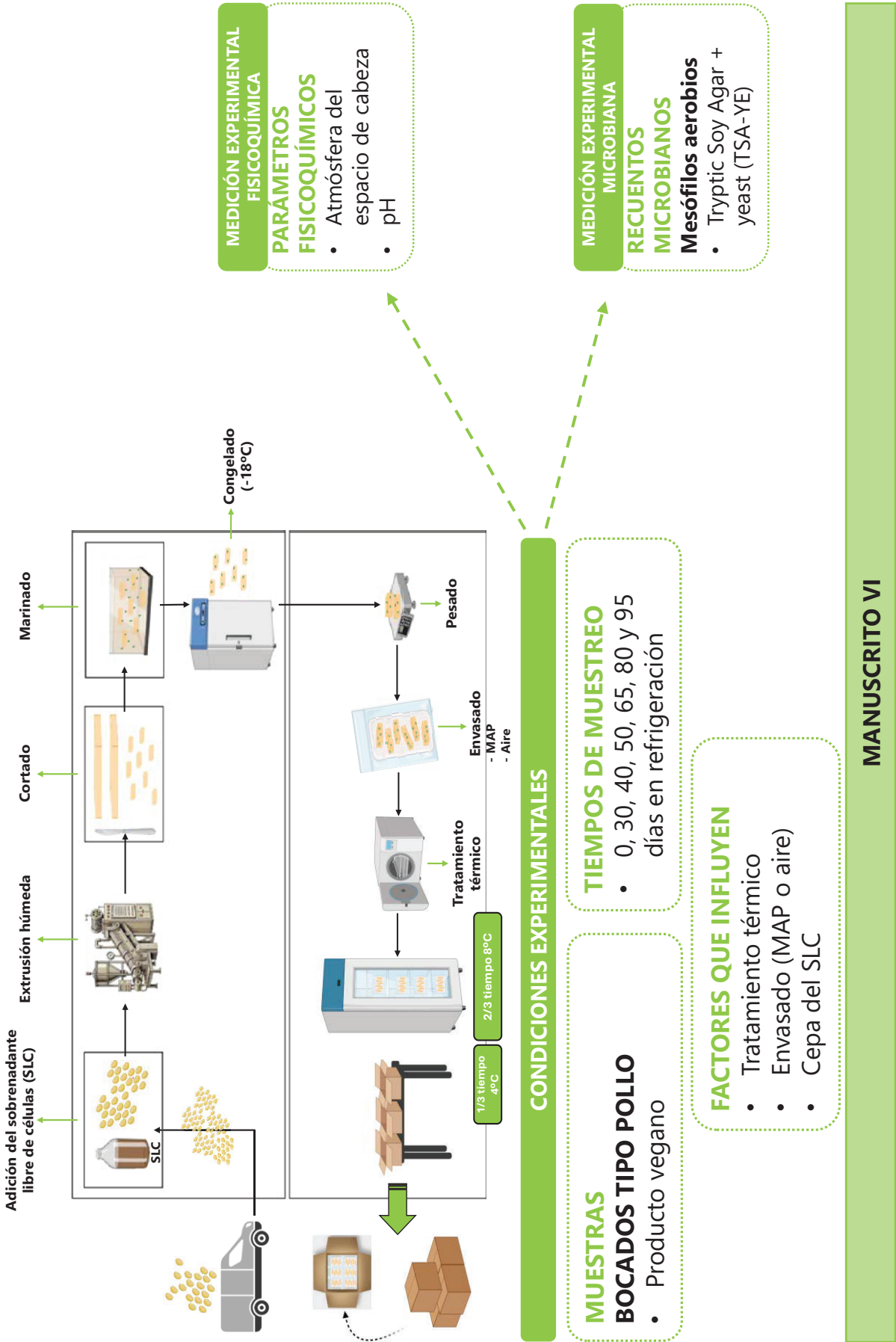
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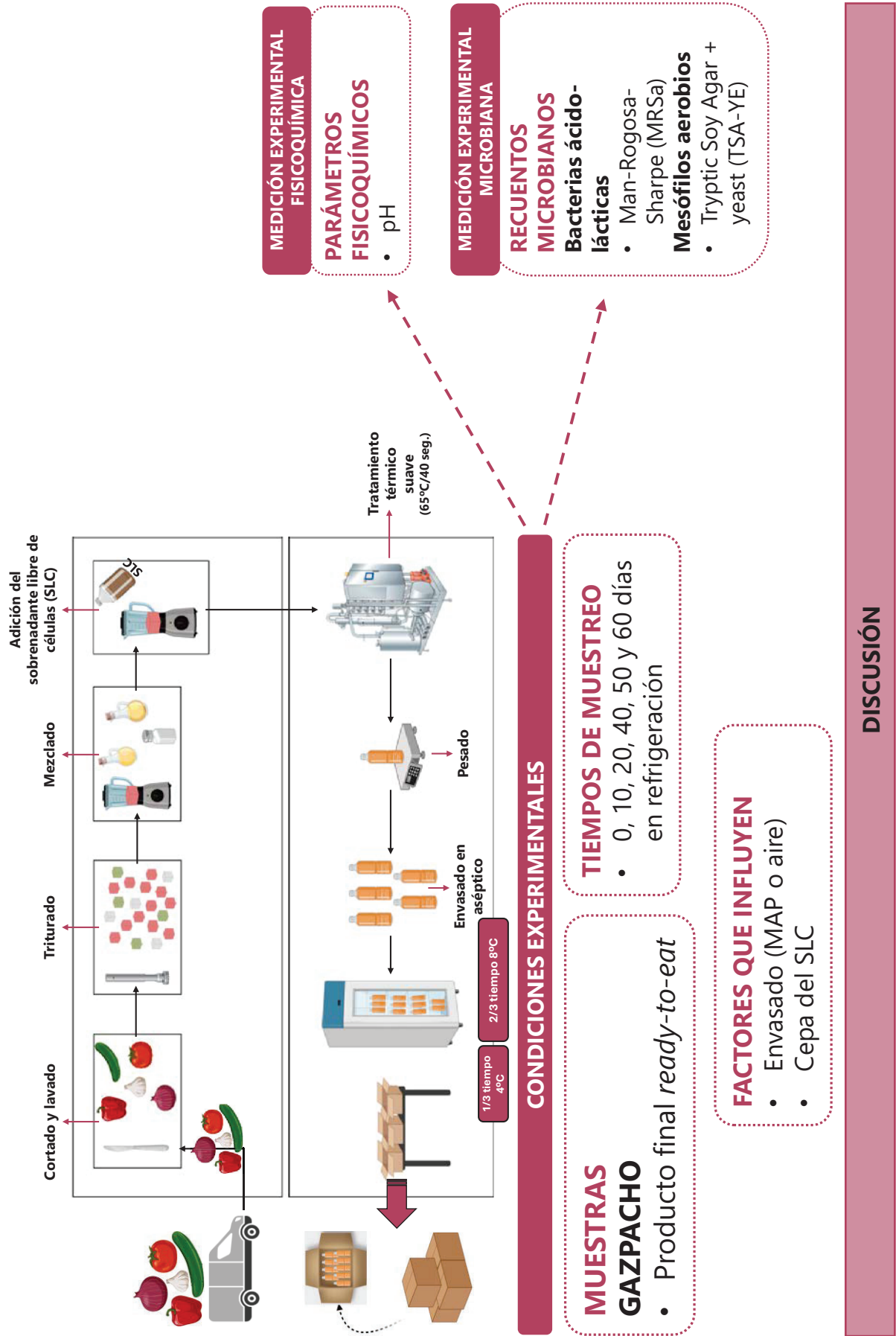
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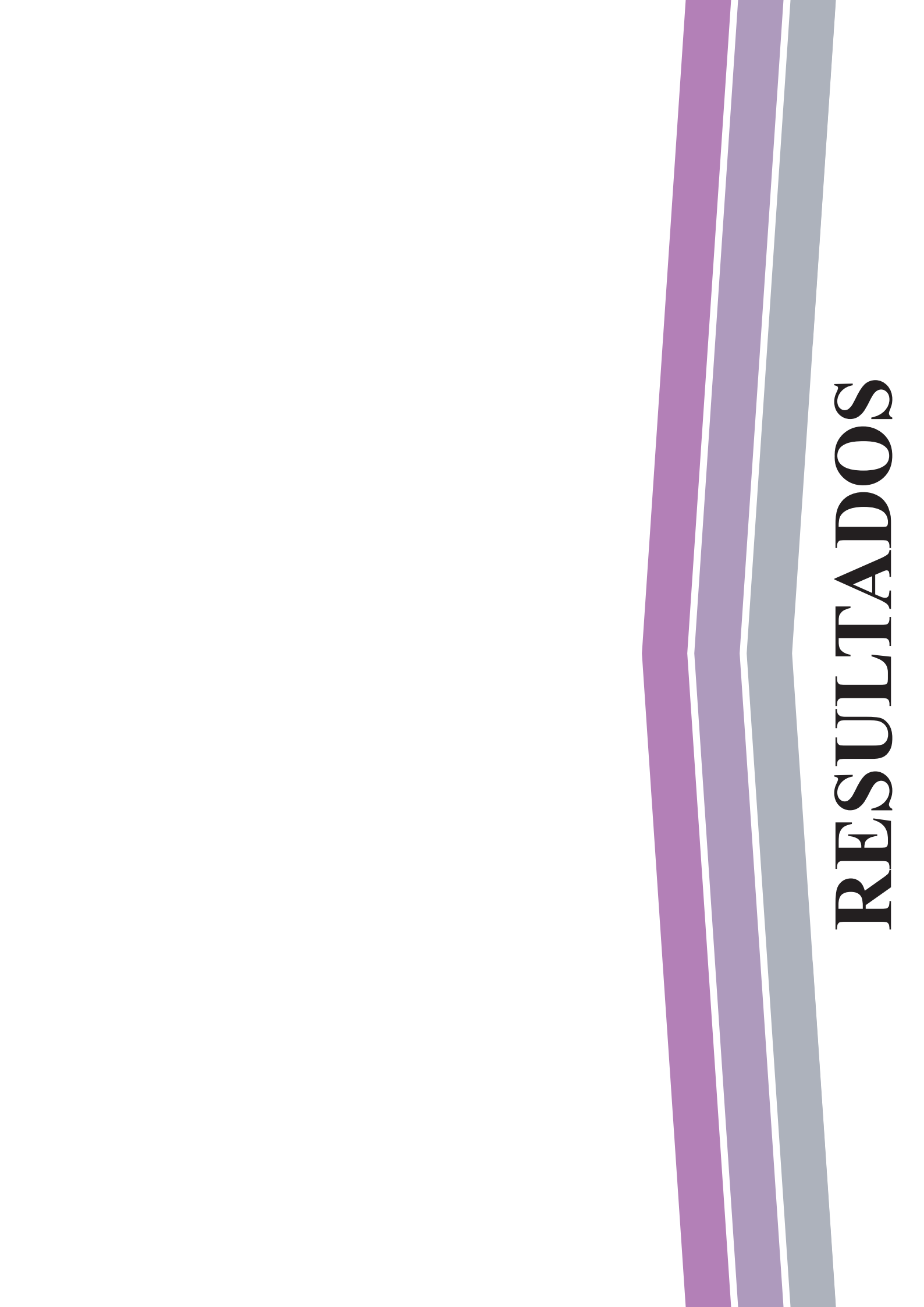
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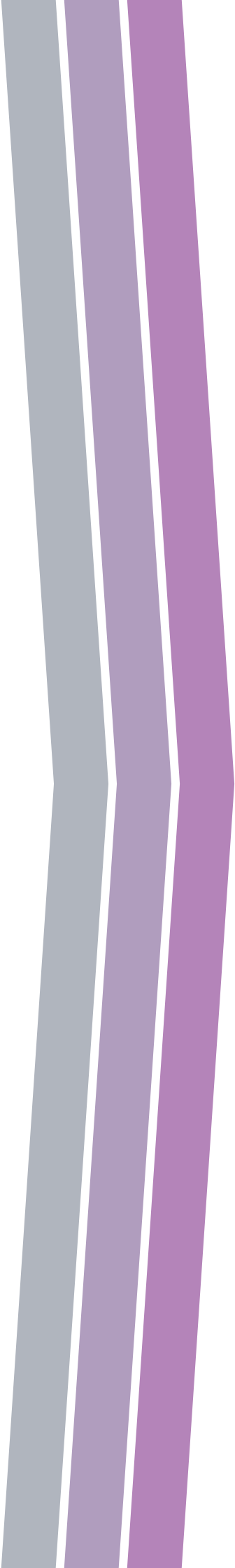








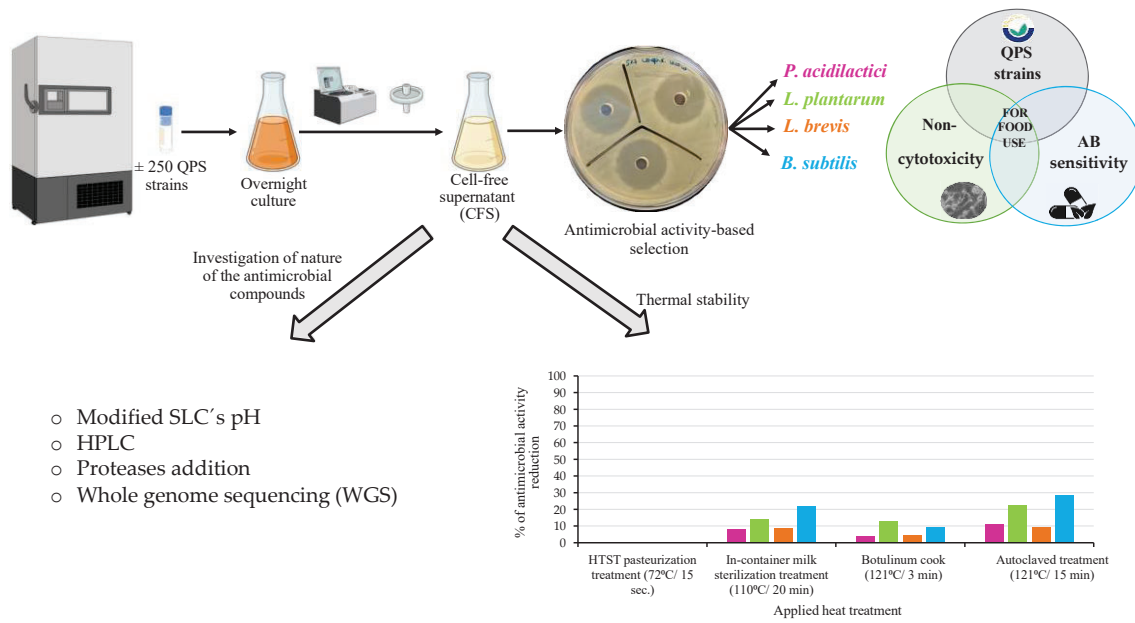
RESULTADOS



Manuscrito III

Lactic Acid Bacteria and *Bacillus subtilis* as Potential Protective Cultures for Biopreservation in the Food Industry

Nerea Garín-Muguialday, Laura Espina, Raquel Virto and Rafael Pagán*



Article

Lactic Acid Bacteria and *Bacillus subtilis* as Potential Protective Cultures for Biopreservation in the Food Industry

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Abstract: The use of bacteria and/or their compounds is an alternative to the use of positive-list additives that the food industry is using as a tool to meet consumer demands for more natural, long-shelf-life, and healthy products, in short, to offer clean label foods. The aim of this study is to investigate the suitability of cell-free supernatants (CFSs) from Qualified Presumption of Safety strains as bioprotective cultures. Out of an initial screening panel of about 200 isolates, strains *Pediococcus acidilactici* CNTA 1059, *Lactiplantibacillus plantarum* CNTA 600, *Levilactobacillus brevis* CNTA 1374, and *Bacillus subtilis* CNTA 517 demonstrated strong antimicrobial activity against, especially, Gram-positive bacteria. The CFSs of these four strains showed minimum inhibitory concentration values between 0.15% and 5% against *Listeria monocytogenes* and *Lentilactobacillus parabuchneri*. None of the four selected strains exhibited acquired resistance to target antibiotics, and the non-toxicity of all the CFSs was demonstrated. In the case of the three lactic acid bacteria, the presence of bacteriocin-like inhibitory substances was confirmed following the decline in antimicrobial activity due to treatment with proteases. Regarding *B. subtilis*, biosynthetic gene clusters for different bacteriocin-like substances, including protease-resistant lipoproteins, were found via whole-genome sequencing. In addition, all of the CFSs exhibited stable antimicrobial activity at a wide range of temperatures (70–121 °C) used for the pasteurization and sterilization of food products, with a loss of antimicrobial activity ranging from 3% to 28%. These results point to the possibility that CFSs from these strains could be used in the food industry as a biocontrol tool to develop new products.

Keywords: biopreservation; protective cultures; lactic acid bacteria; *Bacillus subtilis*; thermoresistance



Citation: Garin-Muguiaday, N.; Espina, L.; Virto, R.; Pagán, R. Lactic Acid Bacteria and *Bacillus subtilis* as Potential Protective Cultures for Biopreservation in the Food Industry. *Appl. Sci.* **2024**, *14*, 4016. <https://doi.org/10.3390/app14104016>

Academic Editor: Anna Lante

Received: 15 April 2024

Revised: 3 May 2024

Accepted: 8 May 2024

Published: 9 May 2024



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1. Introduction

Consumer trends evolve along with technology and science. In this context, more and more consumers are becoming aware of the importance of a healthy and sustainable diet, which transpires to their dietary choices. The rise of “clean label” products epitomizes this trend, emphasizing attributes like additive-free formulae, short ingredient lists, transparent information, and health benefits [1–3]. However, all food has to be safe for consumption, necessitating measures to mitigate the presence of undesirable pathogenic micro-organisms such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. [4]. Additionally, food waste is an additional challenge, particularly due to spoilage micro-organisms like *Penicillium* spp. and *Aspergillus* spp. [5]. Considering this, preservation is a fundamental step in food production processes worldwide, required to guarantee food safety and extend shelf life. On the one hand, conventional preservation technologies employed by the food industry encompass various methods, including heat treatments (such as pasteurization and sterilization), cooling techniques (like refrigeration and freezing), the reduction in water activity (such as spray-drying or freeze-drying), fermentation, and the use of chemical preservatives [6]. Nonetheless, most of these traditional approaches

commonly modify both the nutritional and organoleptic properties of food products, so they are not fully aligned with current consumer preferences and trends [7].

A variety of novel preservation strategies have arisen in response to the clean-label production trend, such as the use of bioprotective bacterial cultures and their metabolites [8–10]. This entails the use of micro-organisms and their products (such as their cell-free supernatants, CFSs) to increase the shelf life of food through competitive exclusion or the production of antimicrobial substances [9,10]. Only microbial species included in the Qualified Presumption of Safety list (QPS) [11] can be used to produce bioprotective cultures, and bacteria should not harbor any acquired transferrable antimicrobial resistance genes to clinically relevant antimicrobials to avoid horizontal transfer to other bacteria [12]. Additionally, and in line with EFSA's guidance on the characterization of production micro-organisms used in feed [13], there should be evidence of the non-toxicogenicity of the cultures and of the stability of the intended technological effect, along with the definition of the quantity to be used at safe levels [10,14,15].

Among possible protective micro-organisms, lactic acid bacteria (LAB) stand out as a compelling choice: they boast a well-established tradition in food applications, are perceived as safe for human consumption, and exhibit a broad spectrum of antimicrobial activity [10]. The antimicrobial capacity of LAB is attributed to the synthesis of various antimicrobial compounds, such as organic acids, hydrogen peroxide, and bacteriocins [16]. Organic acids result from the fermentation of carbohydrates within the surrounding medium or matrix. This generates an adverse environment, preventing microbial proliferation and growth [17]. In aerobic conditions devoid of intracellular catalase, pseudocatalase, and peroxidase, LAB can produce hydrogen peroxide (H_2O_2). This compound leads to the degradation of cellular components, including membrane proteins (SH group oxidation) [16], and the infliction of DNA damage upon the targeted micro-organism [18]. Lastly, bacteriocins, which are ribosomal peptides, exhibit antimicrobial activity whose spectrum varies depending on the specific type of bacteriocin. Additionally, they have demonstrated notable thermoresistance and resilience to pH modifications [19].

In addition to LAB, interest in the genus *Bacillus* has grown due to its multiple biological activities, including antifungal and antimicrobial activity [20]. They are considered QPS strains [11] able to produce different compounds, including bacteriocins. These have been classified depending on their size and post-translational modification in a different classification to the bacteriocins of LAB [21]. The mechanisms of action of these bacteriocins are diverse, among which include the induction of membrane permeabilization in target micro-organisms through the release of enzymes and antimicrobial compounds, inducing alterations in the plasma membrane responsible for delineating the intracellular compartment from the extracellular environment; impeding vital cellular survival processes upon plasma membrane traversal; and intervening in the quorum sensing process associated with pathogen virulence attributed to the motility, biofilm formation, and cell reproduction [22]. Nevertheless, a substantial need for comprehensive information regarding the safety and spectrum of activity is required for its utilization [23] because their activity varies depending on the structure [20].

The main objectives of this work were (i) to select QPS-compliant species from our microbial collection that can produce cell-free supernatants (CFSs) with antimicrobial activity, (ii) to study whether the selected strains are free of acquired antibiotic resistance and are not cytotoxic, (iii) to elucidate the nature of their antimicrobial activity, and (iv) to assess their suitability for application in foods that are subjected to pasteurization or sterilization processes.

2. Materials and Methods

2.1. Selection of Potential Bacterial Strains as Protective Cultures

The CNTA (Centro Nacional de Tecnología y Seguridad Alimentaria) collection encompasses 200 bacterial strains of 24 bacterial species as potential protective cultures, including *Bacillus* sp. (1 species), *Bifidobacterium* sp. (4 species), and lactic acid bacteria

(LAB) (19 species) (Table S1). All of them belong to the QPS list and their identity has been individually verified via 16S rRNA gene sequencing. Table S1 also includes the origins of isolation of each genus.

The assays of the antimicrobial activity of the protective cultures were carried out using the CFSs of the cultures, which were obtained under optimal growth conditions for each micro-organism (Table S2).

To obtain the CFSs, the cultures were centrifuged at $4347 \times g$ for 10 min at 4 °C and the supernatants were filtered with 0.22 µm filters (VWR, Radnor, PA, USA). Then, the CFSs were stored at 4 °C [24,25].

2.1.1. Antimicrobial Activity of the CFS by Agar Well Diffusion Assay

Nine micro-organisms, both pathogenic and spoiling, including different bacteria and yeasts, were selected as indicator micro-organisms (Table S3). In the case of pathogenic micro-organisms, the decision was taken based on the information collected in Rapid Alert System Feed and Food on the main pathogens responsible for food alerts in Europe in 2023 [4]. Spoilage micro-organisms were chosen among the most common micro-organisms in fresh and minimally processed foods, according to CNTA experience.

The antimicrobial activity of the potential CFSs against the indicator strains was determined by using the modified agar well diffusion assay [26]. Briefly, TSA-YE agar (0.8%) was autoclaved, poured 20 mL onto each petri dish (VWR) of 90 mm, and solidified. When the plates were ready, a concentration of 10^6 CFU/mL of the indicator micro-organism was evenly added and spread with a sterile Digrafsky loop to form lawn growth cells. Then, wells of 7.2 mm in diameter were made in the plates using a sterile borer, and the wells were filled with 100 µL of the testing CFS (Table S1). The plates were kept for 2 h at room temperature on the bench to aid the diffusion. Finally, they were incubated in aerobic or anaerobic conditions at different temperatures for 24 or 48 h depending on the micro-organism tested. After that time, the inhibition halo was measured. Considering the criteria outlined by Lesteri et al. [27] and Imade et al. [28], the antimicrobial activity of the CFSs was classified based on the inhibition halo showed by indicator micro-organisms when exposed to each CFS. Including the diameter of the well (7.20 mm), it was deemed negative when the resulting halo was less than 9.20 mm above the diameter of the well, while a halo ranging from 9.20 to 15.0 mm was considered medium activity. If the inhibition halo ranged from 15.00 mm to 25.00 mm, it was categorized as high antimicrobial activity. A halo of more than 25.0 mm was considered as strong antimicrobial activity.

2.1.2. Minimum Inhibitory Concentration (MIC)

To further characterize the antimicrobial activity exhibited by CFS of the selected strains, and to know the dynamic interaction and the antimicrobial activity over time of each of the CFS against the indicator micro-organisms *L. monocytogenes* and *L. parabuchneri*, the time–kill curve was determined, by which the MICs were also obtained. For this purpose, a sterile 96-well flat-bottom microtiter plate (DELTALAB) with increasing concentrations under 10% of the CFSs (0%, 0.15%, 0.31%, 0.625%, 1.25%, 2.5%, and 5%) were used which were confronted to a final well concentration of 10^5 CFU/mL of the indicator micro-organism, either *L. monocytogenes* or *L. parabuchneri*. The plates were incubated at the optimum growth conditions of the target micro-organism and were monitored visually and using a plate reader (mod. EPOCH 2 NS, Agilent, Santa Clara, CA, USA) at a wavelength of 620 nm every 2 h. Finally, the MIC was established as the minimum concentration of CFS necessary to inhibit the growth of the target micro-organism.

2.2. In Vitro Determination of Antibiotic Resistance

The antibiotic resistance of selected isolates was determined by the modified ISO 10932/IDF 223 standard [29] for the Determination of the Minimal Inhibitory Concentration (MIC) of antibiotics applicable to bifidobacterial and non-enterococcal LAB using the microdilution method. MICs were determined for nine antibiotics: kanamycin (Acros Or-

ganics Bvba, Geel, Belgium), gentamicin, streptomycin, tetracycline, clindamycin, chloramphenicol, ampicillin, erythromycin (Merck KGaA, Darmstadt, Germany), and vancomycin (Merck KGaA). The MICs were assessed as the lowest concentration of the antibiotic that inhibited bacterial growth. Based on the MICs, strains were classified as either resistant or susceptible, adhering to the cut-off values and guidelines established by the FEEDAP [30] for LAB and by the SCAN [31] for the *Bacillus* strain.

The values were calculated from three independent experiments and were classified as susceptible (S) when the result was under the cut-off value or resistant (R) when the value was above the cut-off value phenotypes.

2.3. Evaluation of the Cytotoxicity of the Cell-Free Supernatant of *B. subtilis* CNTA 517 on Epithelial Cells

In vitro assessments of the cytotoxicity of the CFS of *B. subtilis* were conducted using Caco-2 and HT-29 cell lines; both were human colorectal cancer cells and were obtained from the Leibniz Institute DSMZ—German Collection of Micro-organisms and Cell Cultures GmbH (DSMZ) (Braunschweig, Germany) collection.

The cytotoxicity was determined by the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck KGaA). The cell lines were seeded in a 96-well flat-bottom microtiter plate at varying concentrations: 5×10^3 cells/well for the Caco-2 cell line and 1.7×10^4 cells/well for the HT-29 cell line. Subsequently, they were incubated at 37 °C, 5% CO₂, and 95% relative humidity for 48 h in the CO₂ incubator SCA-165DRS (ASTEC Co., Ltd., Fukuoka, Japan). In the case of the Caco-2 cell line, the medium was refreshed three times per week. After 15 days of growth to allow cell differentiation into enterocytes, *B. subtilis* CFS was introduced at varying concentrations (5% and 20%). For the HT-29 cell line, the concentrations of 5%, 10%, and 20% of *B. subtilis* CFS were added after 48 h of incubation. The entire volume of cell supernatants was transferred to 96-well U-bottom microtiter plates for subsequent analysis and stored at −80 °C. Afterward, the culture medium was removed and 200 µL of phenol red-free medium (Thermo Fisher Scientific, Waltham, MA, USA) and 50 µL of MTT at a concentration of 5 mg/mL to each well were added. These plates were then placed at 37 °C, 5% CO₂, and 95% relative humidity in the incubator for 3 h until the observation of thread formation under the inverted microscope ECLIPSE TS100/TS100F (Nikon MicroscopyU, Melville, NY, USA). When the established time had elapsed, the culture medium was removed to eliminate unmetabolized MTT. Finally, 150 µL/well of dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific) was added and agitated at 347.76 g for 5 min to dissolve the formazan deposits produced. Then, 100 µL of the supernatant was transferred to a new 96-well flat-bottom plate (DELTALAB, Barcelona, Spain), and the optical density (OD) was measured at a wavelength of 570 nm using the plate reader HEALES, MB-580 (Shenzhen Heales Technology Development Co., Ltd., Shenzhen, China). The cytotoxicity of the CFS of the strain *B. subtilis* CNTA 517 was measured by the viability of the cell lines used following Equation (1):

$$\% \text{ viability} = \frac{OD_{570 \text{ sample}}}{OD_{570 \text{ control}}} \times 100 \quad (1)$$

With this percentage as a basis, statistical significance studies were conducted to ascertain whether there was a decrease in cell cytotoxicity or not.

2.4. Investigation of the Nature of the Antimicrobial Compounds

The antimicrobial activity shown by CFSs could be attributed to various compounds released by the potential protective culture bacteria. Among these, organic acids, hydrogen peroxide, and peptides are commonly the most relevant. To assess whether the antimicrobial activity of the CFSs was due to one or another compound, some assays were carried out in which the indicator micro-organisms were *L. monocytogenes* and *L. parabuchneri*.

2.4.1. Synthesis of Organic Acids and Their Contribution to Antimicrobial Activity

To ascertain the concentration of organic acids (lactic and acetic) and phenylactic acid produced by the selected strains, HPLC was employed. This allowed us to identify the strain that exhibits higher production of either acid under the specified growth. Additionally, to elucidate the influence of the acids on the antimicrobial activity, the stability of the CFSs at different pH levels was determined.

Organic Acids Concentration by HPLC

The concentrations of lactic acid and acetic acid in the samples were quantified using the e2695 high-performance liquid chromatography (HPLC) system (Waters Alliance, Framingham, MA, USA), which was equipped with an Aminex Column HPX-87H 300×7.8 mm (BIO RAD, Berkley, MI, USA) along with its precolumn Micro-Guard Cation H refill cartridges, 30×4.6 m (BIO RAD). The run time was 30 min per sample with an injected volume of 10 μ L. The mobile phase used was 0.005 M H_2SO_4 at a flow rate of 0.6 mL per min. The retention times were 12.564 min for lactic acid and 14.783 min for acetic acid. Detection was carried out using the visible ultraviolet detector PDA 210 nm (2996) (Waters Alliance), following the internal standard method.

In the case of phenylactic acid, its concentration was specifically determined using the method outlined by Zheng et al. [32]. The HPLC system employed remained consistent with the previously mentioned e2695 system (Waters Alliance), while the chromatographic column used was CORTECS C18 2.7 micron (100×4.6 mm) (Waters Alliance). The retention time was 6.20–6.40 min.

Two measurements of each acid concentration were performed on the same day.

Antimicrobial Activity of the Cell-Free Supernatants at Different pHs

To indicate whether the antimicrobial activity was attributable to the presence of organic acids, the pH of the CFS samples was acidified (pH 3.50) and neutralized (pH 7.00), being lower or higher than the pKa of the different expected acids, respectively (pKa of lactic acid at 3.80; pKa of phenylacetic acid at 4.30; and pKa of acetic acid at 4.76). pH adjustments were achieved through the addition of 1 M NaOH or 1 M HCl [33,34]. The antimicrobial activity of the pH-modified CFS against the indicator micro-organisms was determined by the agar well diffusion assay described above, using the untreated CFS as a reference.

If the halo disappeared when neutralizing the CFS, it suggested that the antimicrobial activity was attributed to the organic acids produced by the bacteria. In contrast, if no significant differences were observed ($p > 0.05$), it indicated that the antimicrobial activity of the CFS remained consistent across different pH levels. This suggested that the antimicrobial activity primarily stemmed from the production of a compound other than organic acids [35].

2.4.2. Bacteriocin-like Substance Production by the Cell-Free Supernatants

To confirm that the antimicrobial activity of the potential protective cultures was associated with proteinaceous compounds related to bacteriocin production, the CFSs were subjected to enzymatic treatment using a mixture of two proteases: proteinase K and pepsin (Merck KGaA) [36]. These were simultaneously added; 25 μ L of proteinase K was mixed with 5 mL of the CFS, while, in the case of pepsin, a 0.003 M phosphate buffer was prepared in advance, from which 1 mL was used to dissolve 1 mg of pepsin. Subsequently, 50 μ L of this pepsin solution was added to 5 mL of the CFS. Finally, the mixture was incubated for 1 h at 37 °C under aerobic conditions and the antimicrobial activity of these protease-treated CFSs was determined by the agar well diffusion method (Section 2.1.1) against the indicator micro-organisms, using as reference the CFS without treatment.

If the halo disappeared after the addition of the protease mix, the production of proteinaceous nature compounds (BLIS or bacteriocin-like substances) could be confirmed. However, if the halo persisted, the nature of the compound remained undetermined. In

such cases, two additional proteases were added to the CFSs at varying concentrations which included protamex (NOVOZYMES, Bagsværd, Denmark) at 0.1, 0.5, and 1 mg/mL, and pancreatin (Merck KGaA) [36] at the same concentration range. These were confronted with the indicator micro-organism using the agar well diffusion assay, and the resulting zone of inhibition was quantified. In cases where no halo was observed, signifying a lack of antimicrobial activity in the CFS, it served as evidence of the proteinaceous nature of the compound. If the halo did not disappear, more assays would be needed.

2.5. Thermal Stability of the Cell-Free Supernatants

The heat resistance of the CFSs was measured to know whether the antimicrobial activity of CFSs would be maintained if they were added to foods that would subsequently receive a pasteurization or sterilization treatment (commonly used by the food industry). These treatments were used as a reference to select the intensity (temperature and time) of the different heat treatments (from 10 to 120 min at 70, 90, 110, and 120 °C).

For low temperatures (70 °C and 90 °C), heat treatments were carried out in a thermoblock (Eppendorf, Hamburg, Germany), and, for high temperatures (110 °C and 121 °C), an oil bath (Leyro Instruments, San Sebastián de los Reyes, Spain) was used. A small portion of CFS was added in preheated liquid to ensure the initial temperature, which was measured by the temperature sensor Checktemp 1 HI98509 (HANNA instruments, Limena, Italia). After the preset intervals, the eppendorfs or vials were removed and introduced in cold water. Afterward, the indicator micro-organisms *L. monocytogenes* and *L. parabuchneri* were exposed to the treated CFSs following the agar well diffusion methodology.

Finally, taking as a reference the halo obtained by agar well diffusion assay (Section 2.1.1) of the untreated CFSs against *L. monocytogenes* and *L. parabuchneri*, a formula (Equation (2)) was applied to determine the percentage of antimicrobial activity maintained by each CFS following exposure to various combinations of temperatures and durations.

$$\% \text{ loss antimicrobial activity} = \frac{\text{halo diameter (mm)}_{\text{before heat treatment}} - \text{halo diameter (mm)}_{\text{after heat treatment}}}{\text{halo diameter (mm)}_{\text{before treatment}}} \times 100 \quad (2)$$

Following the representation of the percentage of the retained antimicrobial activity of the CFS throughout time at each temperature, a linear regression was obtained to estimate the loss of antimicrobial activity of the CFS under commercial heat treatments applied by the food industry.

2.6. Whole-Genome Sequencing of the Strains, Identification of AMR Genes, and Identification of Biosynthetic Gene Clusters

The sequenced genome of the *Bacillus subtilis* CNTA 517 strain was obtained through whole-genome sequencing (WGS) using an Illumina iSeq platform (NASERTIC, Pamplona, Spain). Genomic DNA was extracted from 1 mL of fresh culture using the GenElute™ bacterial Genomic DNA kit (Merck KGaA), and tagged with the Nextera XT DNA library prep kit. The 2 × 150-bp paired-end sequencing reads generated were pre-processed by removing adapters, short reads (<140 bp), and poor quality reads (Phred score < 28) using Trimmomatic v.0.39 [37]. After the quality of the filtered clean reads was checked with FastQC v.0.12.1 [38], de novo genome assembly was performed with Shovill v.1.1.0 [39] using the SPAdes assembler with default parameters. Quast v.5.2.0. [40] was used to evaluate the quality of the assembly. Then, 24 long contigs (>500 base pairs) were generated, with 99.24% of reads successfully mapped to the assembly.

The assembled contigs in FASTA format were screened for antimicrobial resistance genes (ARGs) using the ABRicate v. 1.0.1 tool [41] and the CARD [42,43] and ResFinder [44,45] with a minimum identify and coverage values of 80%. The assembled contigs were additionally screened for mobile genetic elements using the MobileElementFinder v.1.0.3 tool [46,47]. Finally, antiSMASH v.6.1.1. [48] was used to predict the type and location of secondary metabolite biosynthesis gene clusters within the assembled genomes.

2.7. Data Analysis and Representation

NCBI's Taxonomy Browser [49], R [50], RStudio 2023.12.1, and the packages ggplot2 [51] and ggtree [52] were used to visualize the initial screening of all CFSs or the active ones against the selected panel of indicator micro-organisms. GraphPad PRISMA software v. 5.00 was used to represent the rest of the graphs. Statistical analyses for comparing each group of samples with the control group were performed in GraphPad using an independent-sample *t*-test at a significance level of 0.05. Normality was assumed for all samples. Since homogeneity of variances could not be assumed, a Brown–Forsythe ANOVA test with Dunnett's multiple comparisons post hoc tests was applied to compare the lactic acid production of the four tested strains at a significance level of 0.05. The same test was applied to compare the acetic acid production among the LAB strains.

3. Results and Discussion

3.1. Preliminary Selection of Potential Bacterial Strains to Be Protective Cultures and Characterization of Their Antimicrobial Activity

The CFSs of 200 strains of potential protective cultures were subjected to the agar well diffusion assay against the indicator micro-organisms. Figure S1 shows the inhibition zones obtained with CFSs derived from cultures that grew normally. Approximately 60% out of all the CFSs exhibited a certain antimicrobial activity, and those strains are depicted in Figure 1. These selected CFSs were more active against Gram-positive indicator micro-organisms, particularly *L. monocytogenes*, *Bacillus cereus*, and *L. parabuchneri*. The observed inhibition halos ranged from 0.92 to >25 mm. Among all the strains under consideration, three representatives of LAB (*Pediococcus acidilactici* CNTA 1059, *Lactiplantibacillus plantarum* CNTA 600, and *Levilactobacillus brevis* CNTA 1374) and one from *Bacillus* sp. (*Bacillus subtilis* CNTA 517) were chosen due to their heightened antimicrobial activity against *L. monocytogenes* and *L. parabuchneri*, exceeding a 15 mm inhibition halo (Figure 1). These strains were isolated from an asparagus by-product (*P. acidilactici* CNTA 1059), homemade chorizo (*L. plantarum* CNTA 600 and *L. brevis* CNTA 1374), and an altered paint (*B. subtilis* CNTA 517).

3.2. Antimicrobial Activity of the CFS

The efficacy of the CFSs produced by the selected strains as a potential protective culture against various species of pathogenic and spoilage micro-organisms is shown in Figure 1 and with more detail in Table S4. In the case of the pathogenic target strains, the resulting inhibition halo for the CFS of the strain *P. acidilactici* CNTA 1059 against *L. monocytogenes* was 24.53 ± 0.21 mm, indicating robust antimicrobial activity. This observation aligns with the results obtained by Hartmann et al. [9] for their strain IDE0550 against three different *L. monocytogenes* strains. Similarly, the CFS of the selected strain *L. plantarum* CNTA 600 exhibited a strong antimicrobial effect against *L. monocytogenes* (15.27 ± 0.47 mm), consistent with the findings of Vataščinová et al. [53]. A medium antimicrobial activity against *B. cereus* (13.36 ± 0.67 mm) was observed, corroborating similar previous results by Akhtar and Syed [54]. *L. brevis* CNTA 1374 CFS showed no antimicrobial activity against any of the pathogenic strains. This contradicted the findings of Jang et al. [55], who reported the antimicrobial activity of the *L. brevis* KU15153 strain against food pathogens, including *L. monocytogenes*, *S. aureus*, *Salmonella* Typhimurium, and *E. coli*. Such discrepancies may be highlighting the variability in antimicrobial activity among different strains of the same species.

Finally, the antimicrobial activity of CFS *B. subtilis* CNTA 517 was strong against *L. monocytogenes* (28.98 ± 0.07 mm) and against *S. aureus* (19.36 ± 0.07 mm). Different authors have reported the strong antimicrobial activity of purified bacteriocins produced by *B. subtilis* against these two pathogenic micro-organisms. Wei et al. [56] documented the antimicrobial activity of subtilisin JS-4, while Taggar et al. [57] described that of peptide-Ba49. However, the reported activity of these peptides was lower than the antimicrobial activity of *B. subtilis* CNTA 517 described in this paper.

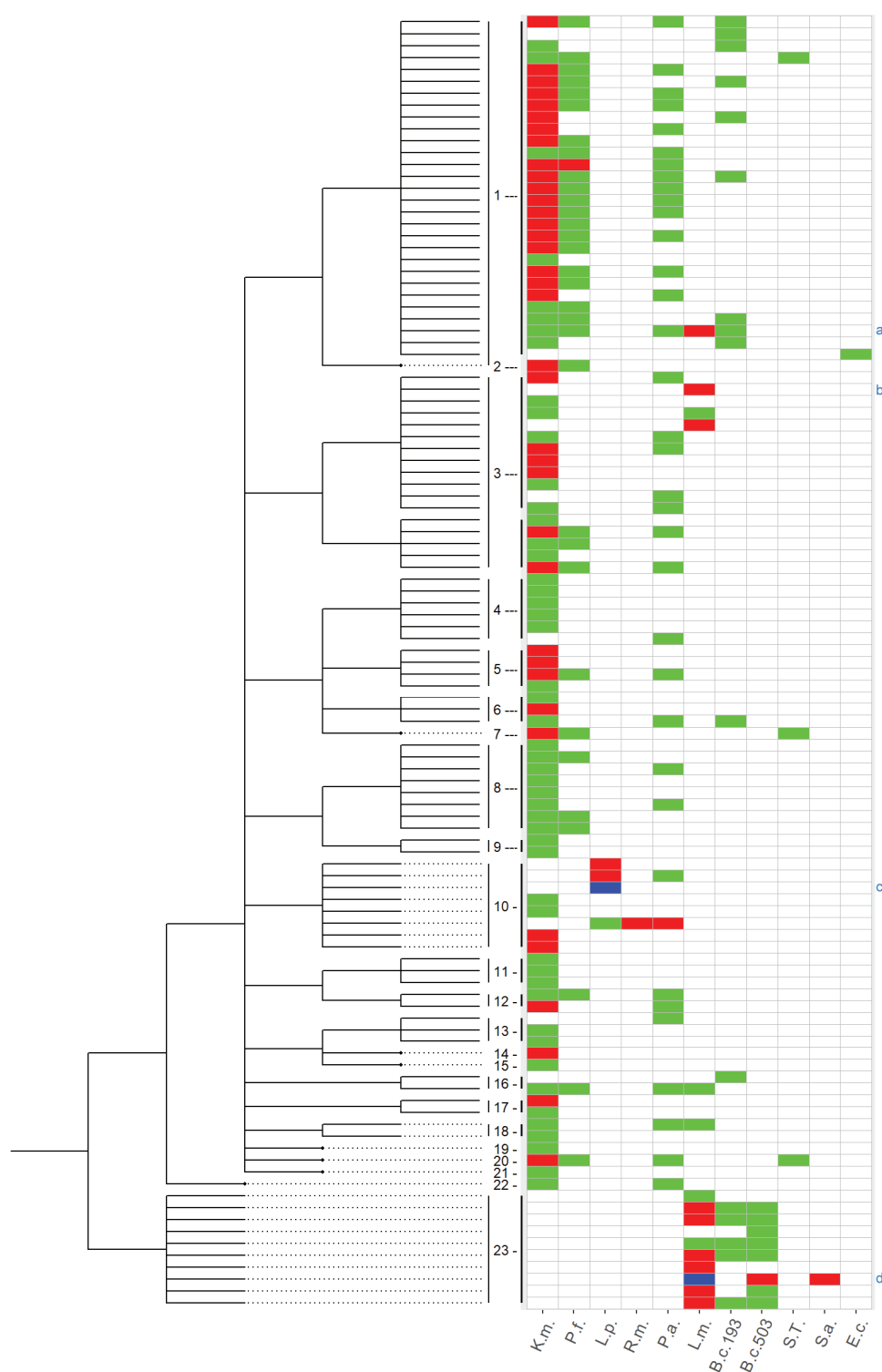


Figure 1. Depiction of the inhibition zone achieved by each selected culture-free supernatant: <9.2 mm (white), $9.2 \text{ mm} \leq$ inhibition zone < 15.0 mm (green), $15.0 \text{ mm} \leq$ inhibition zone < 25.0 mm (red), and $\geq 25.0 \text{ mm}$ (blue). Tested bacterial species: K.m. = *Kluyveromyces marxianus* CNTA.1649, P.f. = *Pseudomonas fluorescens* CNTA 571, L.p. = *Lentilactobacillus parabuchneri* DSMZ 5987, R.m. = *Rothia mucilaginosa* DSMZ 18184, P.a. = *Pseudomonas aeruginosa* CECT 110T, L.m. = *Listeria monocytogenes* CECT 7467, B.c.193 = *Bacillus cereus* CECT 193, B.c.503 = *Bacillus cereus* CNTA 503, S.T. = *Salmonella* Typhimurium CECT 443, S.a. = *Staphylococcus aureus* CECT 976, and E.c. = *Escherichia coli* DSMZ 19206. A dendrogram showing the taxonomic hierarchical relationship between the different tested

strains is also shown. 1 = *Lactiplantibacillus plantarum*, 2 = *Lactiplantibacillus pentosus*, 3 = *Pediococcus acidilactici*, 4 = *Lacticaseibacillus paracasei*, 5 = *Lacticaseibacillus rhamnosus*, 6 = *Lacticaseibacillus casei*, 7 = *Lacticaseibacillus manihotivorans*, 8 = *Limosilactobacillus fermentum*, 9 = *Limosilactobacillus reuteri*, 10 = *Levilactobacillus brevis*, 11 = *Lactobacillus delbrueckii*, 12 = *Lactobacillus johnsonii*, 13 = *Leuconostoc mesenteroides*, 14 = *Leuconostoc citreum*, 15 = *Leuconostoc pseudomenteroides*, 16 = *Latilactobacillus curvatus*, 17 = *Weissella confusa*, 18 = *Companilactobacillus alimentarius*, 19 = *Liquorilactobacillus nagelii*, 20 = *Ligilactobacillus salivarius*, 21 = *Oenococcus oeni*, 22 = *Enterococcus mundtii*, 23 = *Bacillus subtilis*. Selected cultures: a = *Lactiplantibacillus plantarum* CNTA 600, b = *Pediococcus acidilactici* CNTA 1059, c = *Levilactobacillus brevis* CNTA 1374, and d = *Bacillus subtilis* CNTA 517.

The strain *L. monocytogenes* CECT 7467 was selected as the pathogenic indicator micro-organism to be used in the next assays for the CFSs of *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, and *B. subtilis* CNTA 517. In addition to its sensitivity to the CFSs of the strains tested, it is a micro-organism capable of growing in refrigeration and whose presence has accounted for 15% of foodborne illnesses according to the annual report of the RASFF of the year 2023 [4].

The antimicrobial activity of the CFSs from *P. acidilactici* and *B. subtilis* species against the selected spoilage micro-organisms could be classified as negative or null, as they did not produce any halos in the agar well diffusion assay. On the contrary, the CFS of *L. plantarum* CNTA 600 exhibited a medium activity against *P. fluorescens* (10.78 ± 0.05 mm) which aligned with the activity described by Lv et al. [58]. It also demonstrated strong activity against *K. marxianus* (14.14 ± 0.12 mm), for which the antimicrobial activity of different antimicrobials has not been described in the literature yet [59]. Notably, the CFS displaying the highest antimicrobial activity was the one from *L. brevis* CNTA 1374, producing halos of 32.20 ± 0.04 mm against the spoilage LAB *L. parabuchneri*, not previously described. Considering these results and its ability to produce histamine in cheese [60], *L. parabuchneri* CNTA 1075 was selected as the spoilage indicator micro-organism to be tested against the CFS of *L. brevis* CNTA 1374 in the following tests.

The MIC of the CFS of the different species was determined by the time–kill curve and was seen to be specific for each strain but within a similar range (Figure S2). The MICs of *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, and *B. subtilis* CNTA 517 against *L. monocytogenes* were 2.5%, 5.0%, and 1.25%, respectively. In the case of *L. brevis* CNTA 1374, a lower MIC of 0.15% was determined against *L. parabuchneri*. These results align perfectly with those obtained with the agar diffusion assay, which showed *L. brevis* CNTA 1374 to attain the greatest inhibition halo, while *L. plantarum* CNTA 600 showed the weakest activity out of all the selected strains.

3.3. Antibiotic Resistance of the Selected Strains

The MIC was determined by a broth microdilution assay and the results in Table 1 indicated that, among LAB, only *L. brevis* CNTA 1374 demonstrated sensitivity to all antibiotics tested according to the referenced cut-off values [30]. However, it should be noted that kanamycin resistance has been described as intrinsic in pediococci and most lactobacilli species (including *L. plantarum*) [61–63]. This has been observed in numerous previous works and has been explained by the high rate of spontaneous chromosomal mutations conveying resistance to aminoglycosides, having a low potential for horizontal spread [61,64,65].

In the case of *B. subtilis* CNTA 517, Adimpong et al. [66] already pointed out that the cut-off antibiotic values defined by SCAN [31] more closely reflect the intrinsic resistances of *B. subtilis*, in contrast to those set by FEEDAP [30]. In this regard, *B. subtilis* CNTA 517 was deemed susceptible to all tested antibiotics. Furthermore, no mobile genetic elements were found after sequencing this strain, and all the detected antibiotic resistance genes (detailed in Table S5) are also found in the type of strain *B. subtilis* subsp. *subtilis* str. 168 (NCBI Reference Sequence NC_000964.3). These findings support the hypothesis that *B. subtilis*

CNTA 517, like the three tested LAB strains, does not harbor any acquired antimicrobial resistance which could be transferred to other bacteria.

Table 1. Antibiotic resistance of the selected strains according to the broth microdilution assay. The S/R values were taken following the FEEDAP cut-off values for *Pediococcus* spp., *Lactobacillus plantarum/pentosus*, *Lactobacillus* obligate heterofermentatives [30], and SCAN values for the *Bacillus* sp. [31].

Potential Protective Culture	Antibiotic Resistance *								
	Resistant/Sensitive								
	GEN	KAN	STR	TET	CLN	CHL	AMP	ERY	VAN
<i>Pediococcus acidilactici</i> CNTA 1059	S	R	S	S	S	S	S	S	n.r.
<i>Lactiplantibacillus plantarum</i> CNTA 600	S	R	n.r.	S	S	S	S	S	n.r.
<i>Levilactobacillus brevis</i> CNTA 1374	S	S	S	S	S	S	S	S	n.r.
<i>Bacillus subtilis</i> CNTA 517	S	S	R	S	S	S	n.r.	S	S

* GEN: gentamycin; KAN: Kanamycin; STR: Streptomycin; TET: Tetracycline; CLN: clindamycin; CHL: chloramphenicol; AMP: ampicillin; ERY: erythromycin; VAN: vancomycin. S: sensitive to antibiotic; R: antibiotic resistant; n.r.: not required.

3.4. Evaluation of the Cytotoxicity of the Cell-Free Supernatant of *B. subtilis* CNTA 517 on Epithelial Cells

The effect of different concentrations of the CFS of *B. subtilis* CNTA 517 on Caco-2 and HT-29 (Figure S3) cell lines was determined by the MTT method. At the assayed conditions, the concentrations of the tested CFSs were found to be non-toxic. These results coincide with the research presented in the works of Brutscher et al. [67] and Chen et al. [68], affirming the non-toxicogenic characteristics of this micro-organism in epithelial cells. According to these observations, the CFS of the strain *B. subtilis* CNTA 517 could, therefore, be considered as an ingredient for industrial use in food.

3.5. Nature of the Antimicrobial Compounds Contained in CFS

The nature of the antimicrobial compounds produced by the bacteria could be of organic origin, so the organic acid-producing capacity of the selected strains was determined. On the other hand, the change in pH of the CFS was related to the antimicrobial activity they showed, and it was possible to determine whether it was due to the production of these compounds or to the production of antimicrobial peptides.

3.5.1. The Synthesis of Organic Acids and Their Contribution to Antimicrobial Activity

In a first step, the concentration of organic acids was measured by HPLC. Lactic acid and acetic acid stand out as the two most prevalent organic acids generated by LAB [69]. Table 2 shows the concentration of the organic acids of each CFS. Concerning lactic acid, *P. acidilactici* CNTA 1059 exhibited the highest production yielding 8.20 ± 0.19 g/L, similar to the previously described for another *P. acidilactici* strain [70]. In contrast, *B. subtilis* CNTA 517 demonstrated the lowest lactic acid production, reaching a value of 2.70 ± 0.17 g/L, which is lower than the 3.63 g/L observed for other *B. subtilis* strains [71].

Table 2. Organic acids concentration on the cell-free supernatants (CFSs). Different superscripts indicate statistical differences among the mean lactic acid or acetic acid production of the tested strains.

Strain	Organic Acids (g/L)		
	Lactic Acid	Acetic Acid	Phenylactic Acid
<i>Pediococcus acidilactici</i> CNTA 1059	$8.20^a \pm 0.19$	$3.58^x \pm 0.07$	119.13 ± 1.17
<i>Lactiplantibacillus plantarum</i> CNTA 600	$7.39^a \pm 0.13$	$3.64^x \pm 0.00$	-
<i>Levilactobacillus brevis</i> CNTA 1374	$2.80^b \pm 0.02$	$3.78^x \pm 0.07$	-
<i>Bacillus subtilis</i> CNTA 517	$2.70^b \pm 0.17$	<0.25	-

The maximum production of acetic acid was observed by strain *L. brevis* CNTA 1374 amounting up to 3.78 ± 0.07 g/L, surpassing the levels reported by some authors [72]. Notably, *B. subtilis* CNTA 517 did not exhibit detectable levels. Lastly, *P. acidilactici* CNTA 1059 demonstrated a high production capacity, yielding 119.13 ± 1.17 mg/L, which was aligned with the production described by Bustos et al. [70] of 117.7 ± 1.25 mg/L.

Secondly, the pH of the CFSs was modified to elucidate whether the measured organic acids were partially responsible for the antimicrobial activity. Table 3 shows the native pHs of the tested CFS, alongside the antimicrobial activity of the CFS of the four microorganisms selected under pH 3.50 and 7.00. Acidifying or neutralizing the CFS of strain *P. acidilactici* CNTA 1059 did not affect the native antimicrobial activity (24.53 mm). This aligned with the results of Liu et al. [73] regarding the stability of the antimicrobial activity of another *P. acidilactici* strain. Similarly, the CFS of *L. brevis* CNTA 1374 also showed stable antimicrobial activity (compared to the original 32.20 mm of halo size) when its pH was modified, consistent with findings by Sreedharan et al. [74]. In contrast, a significant decrease in antimicrobial activity (from 22.35 mm of original halo size) was observed when acidifying or neutralizing the CFS of *L. plantarum* CNTA 600. This agrees with the results reported by Akhtar and Syed [54] for a *L. plantarum* strain isolated from yogurt. The CFS from *B. subtilis* significantly decreased its native antimicrobial activity (28.52 mm) under acidic conditions, similar to what was previously observed by Epparti et al. [75]. From these results, it could be inferred that the antimicrobial activity of CFSs is not exclusively due to the production of organic acids, and further research is needed.

Table 3. Antimicrobial activity of the cell-free supernatants (CFSs) at pH 3.50 and 7.00.

Strain	CFS pH	Halo Diameter (mm)	
		CFS pH 3.50	CFS pH 7.00
<i>Pediococcus acidilactici</i> CNTA 1059	4.33	23.27 ± 0.72	23.10 ± 0.56
<i>Lactiplantibacillus plantarum</i> CNTA 600	4.44	$20.14^* \pm 0.95$	$19.32^* \pm 0.26$
<i>Levilactobacillus brevis</i> CNTA 1374	5.06	31.89 ± 0.46	31.52 ± 0.12
<i>Bacillus subtilis</i> CNTA 517	6.77	$14.93^* \pm 0.01$	28.98 ± 0.08

* Significantly different from native pH halo size ($p \leq 0.05$).

3.5.2. Antimicrobial Peptide Production by the Cell-Free Supernatants

The antimicrobial activity of the CFS after a protease treatment with proteinase K and pepsin was performed by the agar well diffusion assay against *L. monocytogenes* and *L. parabuchneri*. The original antimicrobial activity achieved with the CFSs of the three tested LAB strains was absent when the CFSs were treated with proteases. This suggests that the antimicrobial activity of these three strains is likely attributed to their ability to produce bacteriocins, as observed for other strains of these species. The ability of *P. acidilactici* to produce bacteriocins was supported by the findings of Fugaban et al. [76] that described the production of pediocin PA-1 in both *P. acidilactici* and *P. pentosaceus*. Similarly, Wu et al. [77] substantiated the bacteriocin-producing capacity of *L. plantarum* RUB 1 via bacteriocin-synthesis-related genes, while Thapar et al. [78] and Sreedharan et al. [74] supported the BLIS production in *L. brevis*.

The protease-treated CFS of the *B. subtilis* strain CNTA 517 achieved an inhibition zone diameter of 26.40 ± 0.69 mm, indicating no statistically significant differences ($p > 0.05$) compared to the untreated CFS. A subsequent proteolytic step was conducted on the *B. subtilis* CFS with the enzyme pancreatin and the commercially available protease mix PRO-TAMEX, but no further reduction of the inhibition zone diameter was achieved. Although *B. subtilis* is known to produce over 20 bacteriocins with distinct functional and structural properties [79], in this case, the nature of the antimicrobial activity remained undetermined, and, therefore, the WGS of this strain was performed.

3.5.3. Antimicrobial Compounds Produced by *B. subtilis* Strain CNTA 517

To help elucidate the antimicrobial activity exhibited by *B. subtilis* CNTA 517, gene cluster mining was carried out on its WGS. Table 4 shows the result of the AntiSMASH screening.

Table 4. Identified secondary metabolite regions in the genome (assembled contigs) of *Bacillus subtilis* CNTA 517 using AntiSMASH with strictness ‘relaxed’.

Region (contig:from-to)	Type	Most Similar Known Cluster	Similarity
01:93826-115524	epipeptide	thailanstatin A	10%
01:335810-377228	other	bacilysin	100%
01:380214-401825	sactipeptide	subtilosin A	100%
01:618860-639606	CDPS	pulcherriminic acid	100%
01:922093-973870	NRP-metallophore, NRPS	bacillibactin	100%
02:317794-358891	T3PKS	1-carbapen-2-em-3-carboxylic acid	16%
02:407348-429246	terpene		
02:502119-579867	NRPS, betalactone	fengycin	100%
02:644080-758852	transAT-PKS, NRPS, T3PKS, PKS-like	bacillaene	100%
03:1-21708	NRPS	-	-
03:430506-451309	terpene	-	-
05:199657-265045	NRPS	surfactin	78%
05:395661-418614	sactipeptide, ranthipeptide	sporulation killing factor	100%
10:1-6820	NRPS	pelgipeptin	37%
14:1-1179	NRPS		

This analysis uncovered the presence of gene clusters responsible for the synthesis of several antimicrobial compounds produced by *B. subtilis*: polyketides (subtilosin, bacillaene), the lipopeptide fengycin, the catechol siderophore bacillibactin, and the dipeptide bacilysin. Gene clusters with a lower similarity to known clusters involved in the biosynthesis of the lipopeptides surfactin and pelgipeptin, as well as two unidentified gene clusters coding for non-ribosomal peptides, were also annotated. With the exception of pelgipeptin, all these compounds have previously been reported to be synthesized by diverse *B. subtilis* strains [80]. *B. subtilis*'s lipopeptides have shown enhanced resistance to enzyme proteolysis and stable activity within a pH range of 4.0–12.0 but reduced activity at lower pH [81]. Considering the results obtained with the CFS of *B. subtilis* CNTA 517, the antimicrobial activity of this strain may be attributed, among others, to the production of surfactin and/or a pelgipeptin-like compound. On the other hand, it should be noted that *B. subtilis* CNTA 517 exerted a superior antimicrobial activity when compared to the rest of the tested *B. subtilis* strains (Figure 1). Consequently, the isolation of the pelgipeptin-like compounds and the other two putative peptides for their further characterization would be of great interest to advance the study of bioprotective agents.

3.6. Thermal Stability of the Cell-Free Supernatants

To assess the suitability of these CFSs for application in food products, different pasteurization and sterilization treatments were explored.

Examples of pasteurization treatments are 72 °C/15 s [82] for milk pasteurization or 90–95 °C for 15–30 s (High Temperature Short Time—HTST), a common treatment applied to the stabilization of citric juices [83]. Pasteurization treatments assayed at 70 and 90 °C for even 90 min did not induce a significant ($p > 0.05$) reduction in the antimicrobial activity of the tested CFSs. Therefore, all the CFSs of the four protective cultures could be considered suitable to be added to products subjected to any kind of pasteurization treatment during their production.

In the case of industrial sterilization treatments, three different practices were selected as examples to apply the equations obtained from the laboratory treatments: an in-container sterilization of milk at 110 °C for 20 min, 3 min botulinum cooking at 121 °C (commonly applied to canned food), and an autoclaving processing of 15 min at 121 °C (commonly

applied to some materials in contact with food). Treatments at 110 and 121 °C for 90 min did result in some loss of activity, depending on both the specific treatment and the tested CFS (Table S6). For the in-container sterilization, the estimated loss of antimicrobial activity would range between 8 and 21%, maintaining 79–92% of the activity. For the botulinum cooking, it would result in a loss of activity ranging from 3% to 13%, so 87–97% of the antimicrobial activity would be retained. When the CFSs were autoclaved, the loss of activity ranged between 9 and 28% (Table 5).

Table 5. Estimation of the lost antimicrobial activity of the cell-free supernatants (CFSs) after two examples of commercial sterilization treatments.

Strain	% of Antimicrobial Activity Lost after the In-Container Milk Sterilization Treatment (110 °C/20 min)	% of Antimicrobial Activity Lost after Botulinum Cooking (121 °C/3 min)	% of Antimicrobial Activity Lost after Autoclaved Treatment (121 °C/15 min)
<i>Pediococcus acidilactici</i> CNTA 1059	8.18	3.73	11.06
<i>Lactiplantibacillus plantarum</i> CNTA 600	13.92	13.12	22.17
<i>Levilactobacillus brevis</i> CNTA 1374	8.36	4.73	9.02
<i>Bacillus subtilis</i> CNTA 517	21.67	9.44	28.70

These results confirmed the findings from various authors regarding the thermal stability of the CFSs (or BLIS) of the bacteria when treated at 100 °C. However, they diverged when subjected to treatments at 121 °C. Both Dhanda et al. [84] and Sidek et al. [85] reported no antimicrobial activity after the treatment at 121 °C. This contrasts with what was obtained with the CFS of *P. acidilactici* strain CNTA 1059. The results of the thermal stability of CFS *L. plantarum* CNTA 600 aligned with Zangeneh et al. [86], who described as thermostable the bacteriocin produced by a strain isolated from traditional sourdough even after treatment at 121 °C for 15 min. However, Qadi et al. [87] reported the loss of antimicrobial activity of two *L. plantarum* strains after treatment at 121 °C for 15 min. The thermostability of the strain *L. brevis* CNTA 1374 at 121 °C was not supported by Qadi et al. [87], who did not describe any antimicrobial activity after a treatment of 121 °C for 15 min of another strain of *L. brevis*. Lastly, Hyun et al. [88] described the thermal stability of the BLIS produced by strain *B. subtilis* BSC35 after a heat treatment of 121 °C for 15 min against *C. perfringens*, and Wei et al. [56] highlighted the stability of the activity exhibited by the bacteriocin subtilin JS-4 from *B. subtilis*.

As all CFSs obtained in this study seem suitable for food subjected to any kind of sterilization treatments, the choice among them will depend on the contaminating microbiota (due to their different antimicrobial spectrum), and the properties of the food itself (like pH or sensory characteristics).

4. Conclusions

Based on the *in vitro* tests conducted, the strains *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, *L. brevis* CNTA 1374, and *B. subtilis* CNTA 517 seem to fulfil the necessary requirements to be used as protective cultures. These strains are properly identified to the species level, are listed in the Qualified Presumption of Safety (QPS) list, do not exhibit acquired resistance to target antibiotics, and do not display toxigenicity upon contact with human epithelial cells.

Instead of using the protective cultures, this study proposes the use of the CFS, showing their potential as biopreservatives in the food industry. The antimicrobial spectrum exhibited by the cell-free supernatants of the selected strains was primarily directed against Gram-positive bacteria, encompassing both pathogenic (*L. monocytogenes*) and spoilage (*L. parabuchneri*) micro-organisms.

The observed antimicrobial activity in all cases can be attributed to the production of bacteriocin-like inhibition substances (BLISs) (and/or lipopeptides in the specific case of *B. subtilis* CNTA 517). These compounds demonstrate stability across various pH levels and temperature fluctuations, making them amenable to be used in products subjected

to thermal preservation treatment, even at sterilization temperatures. Nevertheless, it is essential that we note that these results solely depict the *in vitro* capacity, with the subsequent step involving the testing of these CFSs in diverse food matrices to extend shelf life and potentially reduce or eliminate the need for additives or intense treatments. Therefore, further research is imperative to position CFSs as a viable clean-label alternative for food companies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14104016/s1>, Figure S1: Depiction of the inhibition zone achieved by each tested culture-free supernatant; Figure S2: Growth over time of the selected indicator micro-organisms in presence of the CFS; Figure S3: The cytotoxic effect of *Bacillus subtilis* CNTA 517 cell-free supernatant (CFS); Table S1: Potential protective culture species from CNTA collection employed for the preliminary screening; Table S2: Potential protective culture micro-organisms' growth conditions; Table S3: Pathogenic and spoilage micro-organisms chosen as indicator strains and growth conditions; Table S4: Antimicrobial activity of the cell-free supernatants (CFSs) against pathogenic and spoilage strains with a well diameter of 7.20 mm; Table S5. Antibiotic resistance genes identified in *Bacillus subtilis* CNTA 517; Table S6: Antimicrobial activity of the cell-free supernatants (CFSs) after a heat treatment of 110 °C and 121 °C at different times; Table S7. Lost antimicrobial activity of the cell-free supernatants (CFSs) after heat treatments at 110 and 121 °C.

Author Contributions: Conceptualization, N.G.-M., L.E., R.V. and R.P.; methodology N.G.-M., L.E., R.V. and R.P.; software, N.G.-M. and L.E.; formal analysis, N.G.-M., L.E. and R.V.; investigation, N.G.-M. and L.E.; resources, R.V. and R.P.; data curation, N.G.-M., L.E., R.V. and R.P.; writing—original draft preparation, N.G.-M., L.E., R.V. and R.P.; writing—review and editing, N.G.-M., L.E., R.V. and R.P.; supervision, L.E., R.V. and R.P.; project administration, R.V. and R.P.; funding acquisition, R.V. and R.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Gobierno de Navarra, funding allocation G20001-G2100-4701-467300: Contratación personal investigador y tecnológico, doctorados industriales—project BacBiop (0011-1408-2022-000003). This research was also funded by MCIN/AEI/10.13039/501100011033, Grant PID2021-123404NB-I00, by “ERDF A way of making Europe” and by the Government of Aragón (grant Grupo AESA).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding authors. The raw reads were deposited in the European Nucleotide Archive (accession number of the project: PRJEB74905, sample SAMEA115490881).

Acknowledgments: The University, Innovation and Digital Transformation Department, R&D&I Service of the Government of Navarra (Spain) provided N. Garin-Murguialday with a grant to carry out this research.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

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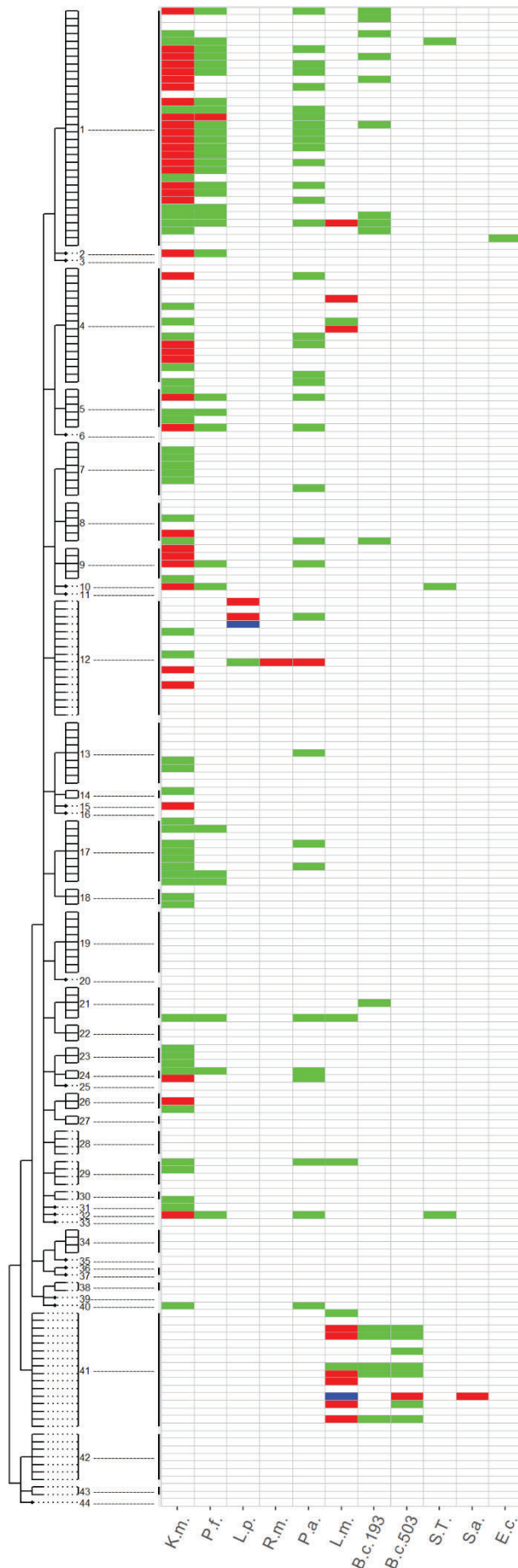


Figure S1. Depiction of the inhibition zone achieved by each tested culture-free supernatant, including the well diameter: < 9.2 mm (white), 9.2 mm ≤ inhibition zone < 15.0 mm (green), 15.0 mm ≤ inhibition zone < 25.0 mm (red), ≥ 25.0 mm (blue).

Tested bacterial species: K.m. = *Kluyveromyces marxianus* CNTA.1649, P.f. = *Pseudomonas fluorescens* CNTA 571, L.p. = *Lentilactobacillus parabuchneri* DSMZ 5987, R.m. = *Rothia mucilaginosa* DSMZ 18184, P.a. = *Pseudomonas aeruginosa* CECT 110T, L.m. = *Listeria monocytogenes* CECT 7467, B.c.193 = *Bacillus cereus* CECT 193, B.c.503 = *Bacillus cereus* CNTA 503, S.T. = *Salmonella* Typhimurium CECT 443, S.a. = *Staphylococcus aureus* CECT 976, E.c. = *Escherichia coli* DSMZ 19206.

A dendrogram showing the taxonomic hierarchical relationship between the different tested strains is also shown. 1 = *Lactiplantibacillus plantarum*, 2 = *Lactiplantibacillus pentosus*, 3 = *Lactiplantibacillus paraplantarum*, 4 = *Pediococcus acidilactici*, 5 = *Pediococcus pentosaceus*, 6 = *Pediococcus parvulus*, 7 = *Lacticaseibacillus paracasei*, 8 = *Lacticaseibacillus casei*, 9 = *Lacticaseibacillus rhamnosus*, 10 = *Lacticaseibacillus manihotivorans*, 11 = *Lacticaseibacillus saniviri*, 12 = *Levilactobacillus brevis*, 13 = *Leuconostoc mesenteroides*, 14 = *Leuconostoc pseudomenteroides*, 15 = *Leuconostoc citreum*, 16 = *Leuconostoc suinucom*, 17 = *Limosilactobacillus fermentum*, 18 = *Limosilactobacillus reuteri*, 19 = *Lentilactobacillus parabuchneri*, 20 = *Lentilactobacillus buchneri*, 21 = *Latilactobacillus curvatus*, 22 = *Latilactobacillus sakei*, 23 = *Lactobacillus delbrueckii*, 24 = *Lactobacillus johnsonii*, 25 = *Lactobacillus acidophilus*, 26 = *Weissella confusa*, 27 = *Weissella cibaria*, 28 = *Loigolactobacillus coryniformis*, 29 = *Companilactobacillus alimentarius*, 30 = *Liquorilactobacillus nagelii*, 31 = *Oenococcus oeni*, 32 = *Ligilactobacillus salivarius*, 33 = *Furfurilactobacillus rossiae*, 34 = *Streptococcus thermophilus*, 35 = *Streptococcus salivarius*, 36 = *Lactococcus lactis*, 37 = *Lactococcus cremoris*, 38 = *Enterococcus faecalis*, 39 = *Enterococcus faecium*, 40 = *Enterococcus mundtii*, 41 = *Bacillus subtilis*, 42 = *Bifidobacterium animalis* subsp. *lactis*, 43 = *Bifidobacterium pseudocatenulatum*, 44 = *Bifidobacterium longum*.

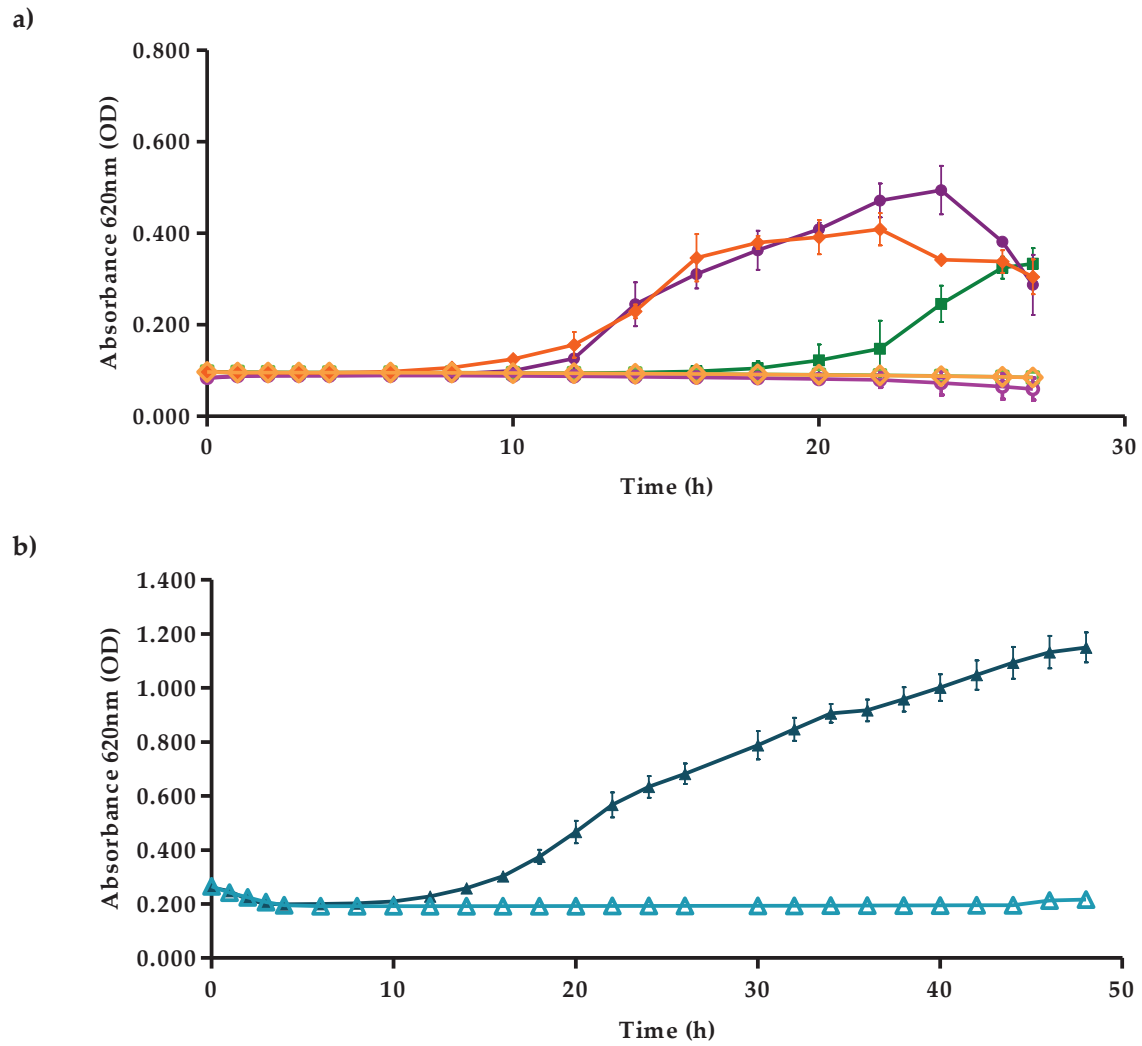


Figure S2. Growth over time of the selected indicator microorganisms *Listeria monocytogenes* (a) or *Lentilactobacillus parabuchneri* (b) measured by absorbance at 620 nm in the presence of cell-free supernatant (CFS) at the maximum non-inhibitory concentration and the minimum inhibitory concentration of each strain: (a) *Pediococcus acidilactici* CNTA 1059 at 1.25% (■) and 2.5% (□), *Lactiplantibacillus plantarum* at 2.5% (●) and 5% (○) and *Bacillus subtilis* at 0.625% (◆) and 1.25% (◇); (b) *Levilactobacillus brevis* CNTA 1374 at 0% (▲) and 0.15% (△) concentration. Data are mean $\pm$ standard deviations (error bars) obtained from at least three independent experiments.

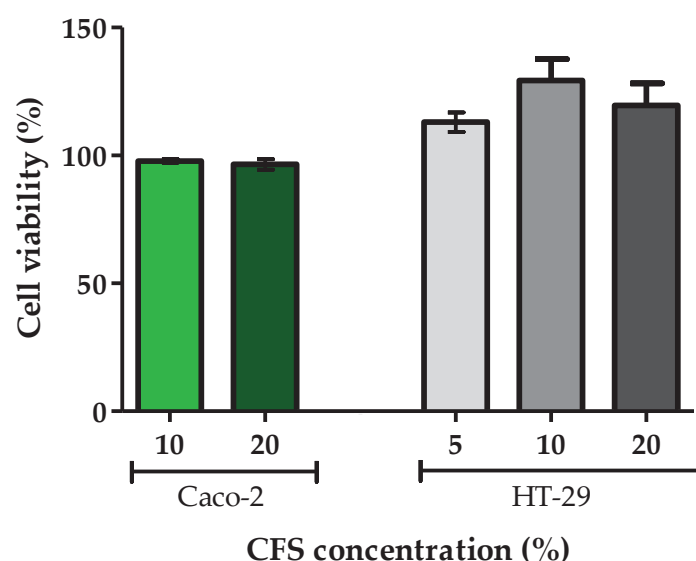


Figure S3. The cytotoxic effect of *Bacillus subtilis* CNTA 517 cell-free supernatant (CFS) on the cell viability of Caco-2 cell line (green colors) at different concentrations of CFS (10% and 20%) and on HT-29 cell line's (grey colors) viability at the CFS concentrations of 5%, 10% and 20%. Data are means $\pm$ standard deviations (error bars) obtained from at least three independent experiments.

Table S1. Potential protective culture species from CNTA collection employed for the preliminary screening.

Potential protective culture species (number of strains)		
<i>Bacillus subtilis</i> (16)	<i>Lactocaseibacillus</i> sp. (24)	<i>Lentilactobacillus</i> sp. (10)
<i>Bifidobacterium longum</i> (1)	<i>Ligilactobacillus</i> sp. (4)	<i>Streptococcus</i> sp. (5)
<i>Bifidobacterium bifidum</i> (1)	<i>Limosilactobacillus</i> sp. (14)	<i>Lactococcus</i> sp. (4)
<i>Bifidobacterium animalis</i> subsp. <i>Lactis</i> (7)	<i>Loigolactobacillus</i> sp. (5)	<i>Enterococcus</i> sp. (7)
<i>Bifidobacterium pseudocatenolatum</i> (2)	<i>Latilactobacillus</i> sp. (8)	<i>Oenococcus</i> sp. (1)
<i>Lactiplantibacillus</i> sp. (39)	<i>Companilactobacillus</i> sp. (4)	<i>Pediococcus</i> sp. (35)
<i>Levilactobacillus</i> sp. (20)	<i>Liquorilactobacillus</i> sp. (2)	<i>Leuconostoc</i> sp. (19)
<i>Lactobacillus</i> sp. (7)	<i>Furfurilactobacillus</i> sp. (1)	<i>Weisella</i> sp. (12)

Table S2. Potential protective culture microorganisms' growth conditions.

Potential protective culture	Growth conditions			
	Medium	Temperature (°C)	Oxygen conditions	Time (h)
Lactic Acid Bacteria	MRSb*	30	Anaerobic	24
Lactic Acid Bacteria	MRSb*	37	Anaerobic	24
<i>Bifidobacterium</i> sp.	MRSb*	37	Anaerobic	24
<i>Bacillus subtilis</i>	TSB-YE**	37	Aerobic	24

*MRSb: Man, Rogosa and Sharpe Broth (Merck GmbH, Darmstadt, Germany)

**TSB-YE: Tryptic Soy Broth (Scharlau, Barcelona, Spain)

Table S3. Pathogenic and spoilage microorganisms chosen as indicator strains and growth conditions.

Pathogenic/ spoilage microorganisms	Growth conditions			
	Medium	Temperature (°C)	Oxygen conditions	Time (h)
<i>Listeria monocytogenes</i> CECT 7467	TSB-YE*	37	Aerobic	18
<i>Escherichia coli</i> O157:H7 DSMZ 19206	TSB-YE*	37	Aerobic	18
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium CECT 443	TSB-YE*	37	Aerobic	18
<i>Staphylococcus aureus</i> CECT 976	TSB-YE*	37	Aerobic	18
<i>Bacillus cereus</i> CECT 193	TSB-YE*	37	Aerobic	18
<i>Pseudomonas fluorescens</i> CNTA 571	PDA**	25	Aerobic	24
<i>Rhodotorula mucilaginosa</i> DSMZ 18184	PDA**	25	Aerobic	24
<i>Kluyveromyces marxianus</i> CNTA 1649	PDA**	25	Aerobic	24
<i>Lentilactobacillus parabuchneri</i> DSMZ 5987	MRSa***	37	Anaerobic	24

*TSB-YE: Tryptic Soy Broth (Scharlau, Barcelona, Spain) (Tunçer and Karaçam, 2020)

**PDA: Potato Dextrose Broth (Scharlau, Barcelona, Spain)

***MRSb: Man, Rogosa and Sharpe Broth (Merck GmbH, Darmstadt, Germany)

Table S4. Antimicrobial activity of the cell-free supernatants (CFS) against pathogenic and spoilage strains with a well diameter of 7.20 mm. Bold typescript indicates the halo size achieved in the selected indicator microorganism.

Indicator strain	Halo diameter (mm)			
	<i>Pediococcus acidilactici</i>	<i>Lactiplantibacillus plantarum</i> CNTA	<i>Levilactobacillus brevis</i> CNTA	<i>Bacillus subtilis</i>
	CNTA 1059	600	1374	CNTA 517
<i>Listeria monocytogenes</i> CECT 7467	24.53 ± 0.21	22.35 ± 0.38	7.20 ± 0.00	28.52 ± 0.08
<i>Staphylococcus aureus</i> CECT 976	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00	19.36 ± 0.07
<i>Salmonella Typhimurium</i> CECT 443	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00
<i>Escherichia coli</i> DSMZ 19206	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00
<i>Bacillus cereus</i> CECT 193	7.20 ± 0.00	13.36 ± 0.67	7.20 ± 0.00	7.20 ± 0.00
<i>Pseudomonas fluorescens</i> CNTA 571	7.20 ± 0.00	10.78 ± 0.05	7.20 ± 0.00	7.20 ± 0.00
<i>Rhodotorula mucilaginosa</i> DSMZ 18184	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00	7.20 ± 0.00
<i>Lentilactobacillus parabuchneri</i> DSMZ 5987	7.20 ± 0.00	7.20 ± 0.00	32.20 ± 0.04	7.20 ± 0.00
<i>Kluyveromyces marxianus</i> CNTA 1649	7.20 ± 0.00	14.14 ± 0.12	7.20 ± 0.00	7.20 ± 0.00

Table S5. Antibiotic resistance genes identified in *Bacillus subtilis* CNTA 517 after screening of contigs after whole genome sequencing for antimicrobial resistance genes using the CARD database (results from the Resfinder database are not shown to avoid duplicities).

Gene	% Coverage	% Identity	Resistance
<i>Bacillus subtilis_mprF</i>	100.00	97.90	Cationic peptides (eg defensins)
<i>vmlR</i>	100.00	96.53	Lincosamide, streptogramin, pleuromutilin, virginiamycin
<i>tet(L)</i>	99.93	80.04	Tetracycline
<i>blt</i>	100.00	99.00	Fluoroquinolone, acridine dye
<i>aadK</i>	99.77	98.83	Aminoglycoside

<i>bmr</i>	100.00	98.97	Fuoroquinolone, nucleoside, phenicol, acridine dye
<i>ykkD</i>	100.00	99.69	Aminoglycoside, phenicol, tetracycline
<i>ykkC</i>	100.00	99.70	Aminoglycoside, phenicol, tetracycline
<i>tmrB</i>	100.00	97.98	Tunicamycin (nucleoside)
<i>lmrB</i>	100.00	99.51	Lincosamide
<i>mphK</i>	100.00	99.13	Macrolide
<i>rphB</i>	97.78	80.33	Rifamycin

Table S6. Antimicrobial activity of the cell-free supernatants (CFS) after a heat treatment of 110 °C and 121 °C at different times, taking into considering the 0.00 mm as 7.20 mm (no halo).

Temperature (°C)	CFS	Halo diameter (mean ± SD mm)				
		0 min	10 min	60 min	90 min	120 min
110	<i>P. acidilactici</i> CNTA 1059	16.73 ± 0.29	15.59 ± 0.33	12.95 ± 0.37	11.93 ± 0.93	9.26 ± 0.93
	<i>L. plantarum</i> CNTA 600	15.13 ± 0.38	12.25 ± 0.19	11.47 ± 0.32	8.08 ± 0.03	4.66 ± 0.61
	<i>L. brevis</i> CNTA 1374	25.00 ± 0.04	22.54 ± 1.48	20.11 ± 0.64	19.75 ± 0.63	17.70 ± 0.20
	<i>B. subtilis</i> CNTA 517	21.78 ± 0.08	17.03 ± 0.61	11.42 ± 1.09	3.75 ± 0.45	0.00 ± 0.00
121	<i>P. acidilactici</i> CNTA 1059	16.73 ± 0.29	14.06 ± 0.42	11.70 ± 0.05	7.99 ± 0.19	2.98 ± 0.73
	<i>L. plantarum</i> CNTA 600	15.13 ± 0.38	10.49 ± 0.60	6.89 ± 0.02	2.97 ± 0.39	0.00 ± 0.00
	<i>L. brevis</i> CNTA 1374	25.00 ± 0.04	22.38 ± 0.45	18.63 ± 0.32	15.53 ± 0.20	13.83 ± 0.47
	<i>B. subtilis</i> CNTA 517	21.78 ± 0.08	16.07 ± 1.53	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table S7. Lost antimicrobial activity of the cell-free supernatants (CFS) after heat treatments at 110 °C and 121 °C.

CFS of	Temperature (°C)	Equation	R ²
<i>Pediococcus acidilactici</i> CNTA 1059	110	0.3432x + 1.333	0.98
	121	0.6105x + 1.9034	0.95
<i>Lactiplantibacillus plantarum</i> CNTA 600	110	0.4975x + 3.9658	0.91
	121	0.7545x + 10.853	0.96
<i>Levilactobacillus brevis</i> CNTA 1374	110	0.2098x + 4.1638	0.92
	121	0.3579x + 3.6514	0.98
<i>Bacillus subtilis</i> CNTA 517	110	0.7987x + 5.7009	0.98
	121	1.6050x + 4.6284	0.99

Manuscrito IV

Bioconservación, alternativa potencial y *clean label* para alargar la vida útil de los alimentos

Nerea Garín-Muguialday, Rafael Pagán y Raquel Virto

Alimentos

INVESTIGACIÓN

BIOCONSERVACIÓN, ALTERNATIVA POTENCIAL Y CLEAN LABEL PARA ALARGAR LA VIDA ÚTIL DE LOS ALIMENTOS

CNTA y la Universidad de Zaragoza, en el marco del proyecto BacBiop, están desarrollando cultivos protectores (bacterias con actividad antimicrobiana) para utilizarlos en diferentes matrices alimentarias (gazpacho, piña, brote de lechuga, queso o análogo de carne), con el objetivo de incrementar la vida útil de estos alimentos, minimizando o sustituyendo el uso de conservantes de la lista positiva de aditivos (E-200s y E-1105). Estos ingredientes se presentan como una alternativa para la industria alimentaria para ofrecer alimentos clean label y con una mayor vida útil comercial.



Diciembre 2023

El desarrollo de nuevas tecnologías de conservación permite avanzar en preservar y aumentar la vida útil de los productos alimentarios y, así, reducir el desperdicio alimentario.

Las tecnologías de conservación son ampliamente conocidas y utilizadas en la industria alimentaria, pero se está observando una actividad investigadora de gran potencial que busca mejorar las soluciones existentes: vidas útiles más largas, menores efectos del uso de la tecnología, etc.

Asimismo, se trabaja e investiga en soluciones más naturales y clean label, una cuestión cada vez más demandada. La consultora Mordor Intelligence estima una tasa de crecimiento anual compuesto (CAGR) entre 2022 y 2028 del 6,75% en la ventas de ingredientes clean label a nivel mundial.

Por ello, una de las tendencias en auge es la utilización de la bioconservación, una tecnología que se presenta como alternativa al uso de aditivos químicos y de la que se prevé a nivel mundial, según Grand View Research, un aumento del mercado en valor del 28,3% CAGR entre los años 2023 y 2030.

Se puede definir la bioconservación como la tecnología que utiliza diferentes microorganismos (o metabolitos que estos producen) como conservantes alimentarios, con el objetivo de mejorar la seguridad alimentaria de alimentos y alargar su vida útil comercial, evitando el uso de aditivos convencionales (los conocidos números E-).

Para conseguir esos microorganismos específicos, en el marco del proyecto BacBiop, financiado por el Gobierno de Navarra, CNTA y la Universidad de Zaragoza (UNIZR) están explorando la combinación de la fermentación de precisión y la bioconservación para identificar, seleccionar y caracterizar cepas bacterianas seguras (QPS) con capacidad de producir compuestos (principalmen-

te bacteriocinas) activos frente a las bacterias patógenas (responsables de la seguridad sanitaria) y frente a las bacterias alterantes (responsables del fin de vida útil) para utilizarse como aditivos en distintos procesos y formulaciones alimentarias.

LOS 4 BIOCONSERVANTES ELEGIDOS

Concretamente, CNTA ha caracterizado la actividad antimicrobiana de 250 cepas bacterianas QPS de distintas especies de la familia de bacterias lácticas y de la especie *Bacillus subtilis* y *Bifidobacterium spp.* Fruto de ello, y considerando las bacterias que mayor actividad antimicrobiana mostraron, se seleccionaron 4 cepas bacterianas de 4 especies: *Lactiplantibacillus plantarum*, *Pediococcus acidilactici*, *Levilactobacillus brevis* y *Bacillus subtilis*.

Además, recientemente CNTA ha conseguido caracterizar la termorresistencia de los sobrenadantes libres de células (SLC), producidos por las 4 cepas seleccionadas, así como su composición de ácidos orgánicos (láctico y acético, principalmente) de bacteriocinas (péptidos ribosomales producidos por bacterias Gram positivas no toxigénicas) y de otros compuestos con actividad antifúngica como, por ejemplo, el ácido fenililáctico.

De acuerdo con los resultados de esta caracterización, CNTA, en colaboración con algunas empresas socias y clientes, ha seleccionado una serie de alimentos y desafíos a superar para usar los bioconservantes propuestos en el proyecto BacBiop, que se pueden observar en la tabla 1.

PRÓXIMOS PASOS

Los próximas actuaciones (que se llevarán a cabo entre 2024 y 2025) que se van a dar dentro del proyecto BacBiop tendrán como objetivo el producir los bioconservantes a partir de las 4 cepas seleccionadas. Esta producción se realizará a través de la tecnología de fermentación de precisión, por lo que se requerirá un cultivo controlado de

TABLA 1

Alimentos y retos en los que CNTA, en el marco del proyecto BacBiop, propone aplicar las 4 cepas bioconservantes.

Alimento propuesto	Reto a resolver
Gazpacho sin pasteurizar	Incremento de vida útil sin modificación organoléptica. No va tratado térmicamente
Brotes de lechuga IV gama (monoproducto)	Incremento de vida útil sin modificación organoléptica.
Piña de IV gama	Desarrollo de un producto nuevo clean label
Mimético de carne incrustado	Reducción de aditivos E-
Queso curado	Reducción del contenido en histamina



cada una de las cepas elegidas para maximizar la producción de los compuestos con actividad antimicrobiana (diferentes en cada una de las cepas).

Cuando se consiga el cultivo completo (biomasa bacteriana + compuestos bioconservantes), dependiendo del uso y alimento, se decidirá si el cultivo vivo se aplica en el alimento. Además, también se tendrá que determinar si se somete a un tratamiento de inactivación para eliminar la actividad metabólica del bioconservante y si se aplica en el alimento, únicamente, los metabolitos termorresistentes (bacteriocinas, ácidos orgánicos y otros compuestos), con actividad antimicrobiana, o se procede a la separación de biomasa y SLC y se utiliza solo la fracción libre de células como conservante.

UNA OPCIÓN INTERESANTE

Los resultados obtenidos hasta el momento dentro del proyecto BacBiop indican que la tecnología de bioconservación es una alternativa con potencial para conseguir vidas útiles más prolongadas, sin utilizar ningún aditivo artificial, en diferentes matrices alimentarias.

En la segunda parte del proyecto, se comprobará la viabilidad de esta tecnología para poder

producir industrialmente bioconservantes para utilizarlos en alimentos como gazpacho, brotes de lechuga, piña, alternativas de carne o queso curado. ■

CNTA
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* Gobierno de Navarra ha financiado la contratación en CNTA de la doctoranda Nerea Garín en el marco de la convocatoria de ayudas 'Doctorados Industriales 2022'.

** El trabajo que se describe en este artículo se está realizando gracias a la ayuda para la realización de Tesis Doctorales del Gobierno de Navarra.

Manuscrito V

Quality and safety preservation of pineapple sticks using the cell-free supernatant of protective cultures

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Article title

Quality and safety preservation of pineapple sticks using the cell-free supernatant of protective cultures

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Abstract

The demand for clean-label and minimally processed foods has increased the interest in natural biopreservation strategies. The present study evaluates the use of the cell-free supernatants (CFSs) of three lactic acid bacteria (LAB) strains (*Pediococcus acidilactici* CNTA 1059, *Levilactobacillus brevis* CNTA 1374 and *Lactiplantibacillus plantarum* CNTA 600) as potential bioprotective cultures to preserve fresh-cut pineapple sticks. Two spoilage yeasts, *Meyerozyma* spp. and *Rhodotorula toruloides*, were identified as the predominant microbiota responsible for deterioration. The CFSs of the three LAB strains were then tested in combination with passive modified atmosphere packaging for their ability to inhibit microbial growth and preserve physicochemical and sensory properties during refrigerated storage. Among the treatments, *P. acidilactici* CNTA 1059 CFS effectively inhibited yeast proliferation while preserving the colour, texture, and flavour of the pineapple sticks for up to 12 days. Consumer-based evaluations further confirmed the improved acceptability and the extension of shelf life of the treated samples compared to controls. The adequacy of *P. acidilactici* CNTA 1059 as a bioprotective strain was supported by whole-genome sequencing analysis, which confirmed the absence of transmissible antibiotic resistance genes and non-pathogenic status, and the presence of a pediocin PA-1 biosynthetic cluster. The predicted production of pediocin was further validated by molecular characterization of the CFS via mass spectrometry. These results present *P. acidilactici* CNTA 1059 as a safe, effective, and multifunctional LAB strain, whose CFS can extend the shelf life of minimally processed pineapple sticks, with potential applications for other minimally processed fruits to meet clean-label demands.

1. Introduction

Contemporary consumer trends are increasingly moving towards healthy, chemical-free, sustainable, or clean-label products, and ready-to-eat (RTE) products (Alegbeleye & Sant'Ana, 2022). According to the European Community Standards, RTE foods are those intended for direct consumption with minimal processing and handling (Botondi et al., 2021). Despite a decline in production during the pandemic years, a compound annual growth rate of 6.5% of RTE products is expected through 2032 (DataIntel, 2023), a period in which the fresh-cut fruit market will continue to increase (Testa et al., 2021).

RTE fruit and vegetables are common products whose processing involves peeling, coring, cutting, which trigger metabolic and enzymatic processes, affecting colour, odour, flavour (Botondi et al., 2021; Iturralde-García et al., 2022), and firmness (Giannakourou & Tsironi, 2021; Iturralde-García et al., 2022). Additionally, processing and cold storage increase nutrient and water release, making them more accessible to microorganisms (Collu et al., 2021). This includes spoilage microorganisms like lactic acid bacteria, moulds and yeasts, and pathogen microorganisms like *Pseudomonas* spp. and members of the *Enterobacteriaceae* family (Giannakourou & Tsironi, 2021; Iturralde-García et al., 2022). These challenges are especially pronounced in RTE perishable fruits such as fresh-cut pineapple, whose quality quickly declines even at refrigeration temperatures (O'Connor-Shaw et al., 1994).

The food industry addresses this issue through meticulous handling, disinfection, and preservation treatments (Paulsen et al., 2023), such as the use of modified atmosphere packaging (MAP) to preserve the product's freshness (Giannakourou & Tsironi, 2021). Disinfection of RTE fruit and vegetables with sodium hypochlorite is commonly carried out in the food industry (Arbor, 2008), but its application is strictly regulated and discouraged in the European Union due to the potential formation of chlorate and other

halogenated by-products with possible harmful effects (Comission Regulation (EU) 2023/915; EFSA CONTAM Panel, 2014).

To avoid the negative effects of hypochlorite and in response to consumer demands, alternative strategies aligned with the clean label concept are being explored. One promising approach is based on the use of protective cultures, that is, microorganisms added to RTE fruits and vegetables to control the presence of pathogenic and spoilage bacteria and fungi (Doukaki et al., 2024; Ramos et al., 2020). Additionally, some of these protective cultures can act as probiotics, providing health benefits to the consumer, like through the addition of live bacteria in edible coatings of strawberries (Khodaei & Hamidi-Esfahani, 2019; Tumbarski Y., 2019), kiwis (Hashemi and Jafarpour, 2021), apples (Álvarez et al., 2021; Todhanakasem et al., 2022), and pineapples (Tenea et al., 2020). Similarly, the incorporation of cell-free supernatants (CFS) from live cultures, rich in antimicrobial metabolites such as organic acids or bacteriocins, offer effective microbial control without the addition of live microorganisms (Barbosa et al., 2017). For this purpose, lactic acid bacteria (LAB) are particularly suitable due to their well-documented antimicrobial activity, widespread use in food (Hernández Figueroa et al., 2024), and the Qualified Presumption of Safety (QPS) status granted to most of their species (EFSA BIOHAZ Panel, 2024).

The main objective of this study was to evaluate the potential of three previously selected CFSs from three LAB strains (*Pediococcus acidilactici* CNTA 1059, *Levilactobacillus brevis* CNTA 1374, and *Lactiplantibacillus plantarum* CNTA 600) as natural antimicrobial agents for extending the shelf life of fresh-cut pineapple. The study followed a stepwise approach: (i) identification of the predominant spoilage microbiota associated with fresh-cut pineapple; (ii) study of the influence of CFS treatment on physicochemical parameters of minimally processed product through preliminary

screening; (iii) evaluation of their antimicrobial potential to select the most effective treatment; and (iv) validation of the selected treatment through testing under abusive temperatures and a consumer-based sensory evaluation. Additionally, the study aimed at the (v) exploration of the underlying mechanisms responsible for the antimicrobial activity of the selected CFS.

2. Material and methods

2.1. Isolation and Identification of Pineapple Spoilage Microorganisms

The fruit utilized in this experiment was pineapple (*Ananas comosus* (L.) Merr.). The first step involved storing the peeled and cut fruit under refrigeration until the spoilage microorganisms were visually observed. At that point, samples were taken for microbiological analysis. To simulate typical marketing conditions for perishable products, the cut fruit was stored at 4 °C/ 4 days (1/3 of the shelf life) and then at 8 °C/ 8 days (2/3 of the shelf life) in a cold room, following the approach described by de Andrade Cavalari et al. (2024). After this 12-day period, the fruit was homogenized under sterile conditions (Biomérieux, Marcy-l'Étoile, France) with 0.1% buffered peptone water (BPW) (Merck KGaA, Darmstadt, Germany). The resulting homogenate was diluted to different concentrations and seeded on various agar media as previously described by Yousuf & Srivastava (2017) for subsequent enumeration and microbial identification (Table S1).

Following the isolation of the colonies, taxonomic identification was performed using 16S rRNA gene sequencing for bacteria and the region D1/D2 of the 26S rDNA gene sequencing for fungi. Genomic DNA was extracted from pure cultures using the silica column commercial kit GenElute™ Bacterial Genomic DNA Kit Protocol (NA2110, Sigma-Aldrich, Massachusetts, United States of America) according to the

manufacturer's instructions. The V1-V3 region of the 16S rRNA gene was amplified using bacterial primers 27F-YM (5'-AGAGTTTGATYMTGGCTCAG-3') and 515R (5'-ATCGTATTACCGCGGCTGCTGGCA-3') (Stackebrandt and Goodfellow, 1991), while the region D1/D2 of the 26S rDNA was amplified using the fungal-specific universal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTCCAAGACGG-3') (O'Donnell, 1993). PCR products were purified using the PCR purification kit mi-PCR Purification Kit (Cat. No. mi-PCR250) (Metabion International AG, Planegg, Germany) and sequenced via Sanger sequencing using an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequences were trimmed, quality-checked, and compared against the NCBI nucleotide database using the BLASTn algorithm to assign taxonomic identity based on sequence similarity (fungi were identified at the species level based on a similarity greater than 97%).

2.2. Preparation of the Cell-Free Supernatants

The selection of the strains to produce the CFSs was based on the results of a previous work (Garin-Murguialday et al., 2024). These microorganisms were *P. acidilactici* CNTA 1059, *L. brevis* CNTA 1374 and *L. plantarum* CNTA 600 of the selected strains and their CFSs were obtained following the methodology previously described (Garin-Murguialday et al., 2024), wherein overnight pre-cultures were centrifuged at $4347\times g$ for 10 min at 4 °C and filtered with 0.22 µm filters (VWR, Radnor, PA, USA).

2.3. Pineapple sticks preparation and storage

The pineapples used in this study were acquired in the same month but from different batches. The pineapples were disinfected with running tap water at 4 °C containing 100 ppm sodium hypochlorite for 3 min, then rinsed in tap water at 4 °C for 5 min and drained. Both coring and peeling were performed manually. The fruit was cut

into 16 sticks (60 g) with a sterile steel knife and subsequently immersed in running tap water refrigerated (4° C) or in the corresponding pure cell-free supernatant (CFS) of *P. acidilactici* CNTA 1059, *L. brevis* CNTA 1374, or *L. plantarum* CNTA 600 for treated batches, which constituted the 5% v/w. Excess solution was drained for 15 min at 4° C.

A portion of the untreated sticks were packaged in TS250 polypropylene trays (Linpac, Featherstone, England) with a capacity of 250 mL, containing one stick per package and these were packed with a macroperforated (Amcor Flexible; Victoria, Australia) film. Another portion of the untreated and all the treated pineapple sticks were packaged in the same type of trays, also individually, but using Passive Modified Atmosphere packaging (MAP). More specifically, they were heat-sealed with a medium-permeability 40 µm thickness microperforated film (Amcor Flexible) using a B160A semi-automatic packaging machine (ORA, Trevoux, France).

Therefore, there were 5 work batches: control-air (C1) (water + air), control-MAP (C2) (water + passive MAP), and CFS of *P. acidilactici* CNTA 1059 CFS + passive MAP, *L. brevis* CNTA 1374 CFS + passive MAP and *L. plantarum* CNTA 600 CFS + passive MAP.

As stated before, the sticks were stored for 1/3 of the time at 4 °C and 2/3 at 8 °C. Trays (5 per day for each batch) were randomly sampled at 0, 4, 8 and 12 days for headspace gas determination, physicochemical determinations (texture, °Brix, acidity, maturity index and CIEL*a*b* colour coordinates), microbial analysis, and sensory evaluation. Additionally, a parallel shelf-life study was included, simulating commercialization/distribution under abusive temperatures. The pineapple sticks were stored at 10°C for 10 days (Iglesias et al., 2018).

2.4. Headspace atmosphere determination and physicochemical analyses

2.4.1. Headspace atmosphere determination

An automatic gas analyser (PBI, Dansensor; Barcelona, Spain) was used to determine the headspace gas composition before the physicochemical analyses at each sampling time in five random trays for each batch. A syringe was inserted through an adhesive septum affixed to the film cover to measure the carbon dioxide and oxygen levels. The results are presented as percentages.

2.4.2. Texture evaluation

The texture analysis of fresh-cut pineapple was conducted over a 12-day period of cold storage using a penetration test. Firmness was determined as the peak value on the Y-axis and expressed per unit of force (N) (Russo et al., 2014). It was assessed using a TA-XT PLUS texture analyzer (Stable Micro Systems, Godalming, England) equipped with a Magness-Taylor penetration probe. The methodology was adapted from Frisón et al. (2021), involving the insertion of a 2 mm diameter stem into three distinct points on each pineapple stick at a velocity of 0.005 m/s, penetrating to a depth of 10 mm. Measurements were taken at each sample time on five samples from each batch.

2.4.3. Maturity index

The maturity index (MI) is defined as the ratio of soluble solids content to titratable acidity ($MI = \text{soluble solids} / \text{acidity}$).

2.4.3.1. Measurement of soluble solids content (°Brix)

The total soluble solids content (°Brix) was determined using the method described in the Official Methods of Analysis of Fruit Juices (AOAC, 1984). At each sampling time, 5 sticks were divided into 3 batches, and a juice was made from each to have 3 replicates. Two drops of these juices were analyzed using a digital refractometer (LLG-Labware, Germany) equipped with an automatic temperature corrector feature. The results were expressed as °BRIX at 20 °C.

2.4.3.2. Determination of titratable acidity

The total content of natural acids was quantified using the potentiometric method (AOAC, 1990) through titration with a sodium hydroxide solution. Each analysis was performed in triplicate on five pineapple sticks. A 10 mL aliquot of juice from each stick was diluted to 100 mL with distilled water and titrated with 0.1 N sodium hydroxide to reach a pH of 8.10. An automatic titrator (CRISON) was employed for the titration. The total acidity, considering the predominant acid in the studied fruits, was expressed as g of citric acid per 100 mL of pineapple juice.

2.4.4. Colour measurement

2.4.4.1. Instrumental colour determination

CROMA METER CR-400 colorimeter (Konika Minolta, Japan) was employed to measure colour at three distinct points on the surface of five pineapple sticks/batch, with each measurement lasting three seconds. Reflection measurements were taken across both the visible and infrared spectra, with a 1 nm interval for the former and a measurement range from 360 nm to 900 nm for the latter. The resulting data were expressed as ΔE , derived from the L^* , a^* , and b^* coordinates in the CIELAB colour space, a standard established by the International Commission on Illumination (CIE):

$$\Delta E_{ab}^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where ΔE : colour difference between day 0 and day 12; ΔL : difference in luminosity between day 0 and day 12; Δa : difference in red-green coordinate between day 0 and day 12; Δb : difference in yellow-blue coordinate between day 0 and day 12.

2.4.4.2. Colour determination via digital image analysis

In addition to instrumental colour determination, a digital image analysis (Arias et al., 2018) was performed in five sticks of each batch per sampling time to assess whether the application of the CFS treatment caused changes in the product's characteristic colour.

The vision system included the lightning system, a digital camera and image processing software.

Regarding the lighting system, to ensure homogeneous conditions, all sticks were illuminated using two halogen lamps (Philips, Eindhoven, The Netherlands) positioned at a 45° angle to the samples and 40 cm above them. The lamps had a colour temperature of 3000 K.

The camera used to take the pictures was a Canon EOS 10d (Amsterdam, The Netherlands) colour digital camera with a 6.3 Mp CMOS sensor and equipped with a 50 mm-lens. The camera was placed 40 cm in front of each sample with an angle between the camera lens and the axis of the light source of approximately 45°. The images were captured manually using a lens aperture of $f = 2.8$ and a shutter speed of 1/2000. No flash or zoom was used, and the images were taken with autofocus, maximum resolution, and stored in JPEG format. The captured images focused on one side of the stick against a white background.

The images were processed consistently with the CellProfiler version 4.2.6. It was employed to enhance image quality, reduce noise, and determine browning area (%) of the pineapple sticks. Through this process, we determined whether the points in the sieved image belonged to the same object. Each object was assigned a colour, and morphometric measurements were obtained separately for each stick. Finally, a calibration was performed to measure the length of elements in pixels across all images, using known dimensions. The area of each identified object (sections of the stick based on colour) was calculated in square pixels. These results were then converted into real units to determine the percentage of browning: $\% \text{ browning area} = (\text{brown colour pixels} / \text{total object pixels}) \times 100$.

2.5. Determination of yeast populations on pineapple sticks

To evaluate the efficacy of the treatments against microbial growth on the developed product and to establish the conditions for its commercial application.

The sticks from the applied treatments were homogenized with a stomacher homogeneizer blender AES Easy Mix (QuestPair, The Netherlands) with 0.1% BPW, and 1:10 serial dilutions were prepared for subsequent surface spreading on Potato Dextrose Agar (PDA) plates. This medium was selected to allow for the growth of the yeasts identified during the preliminary characterization of spoilage microorganisms.

To evaluate the outcomes, the spoilage population (CFU/g) of the control and treated sticks with the CFS at the initial time point and after 12 days were compared. The difference of the population was calculated as the reduction between the population of the spoilage microorganisms (in log CFU/g) between the treated sticks after 12 days of storage (4 °C/ 4 days and 8 °C/ 8 days) and the treated sticks immediately after the CFS treatment (day 0). This same methodology was applied in the shelf-life study that replicates storage or distribution under abusive temperatures, sampling them at 0, 5, and 10 days for microbial analysis.

2.6. *In vitro* antimicrobial activity of the selected CFS by MIC (minimum inhibitory concentration) determination assay

To assess the antimicrobial activity of the selected CFS against *Rhodotorula toruloides* over time, a MIC determination assay was performed. Sterile 96-well flat-bottom microtiter plates (DELTALAB) were prepared with increasing concentrations of CFS (0%, 0.15%, 2.5%, and 5%) in potato dextrose broth. Each well was inoculated with *R. toruloides* to a final concentration of 10^5 CFU/mL. Plates were incubated under the optimal growth conditions for the yeast, and microbial growth was monitored both visually and spectrophotometrically. Optical density readings at 620 nm were recorded

every 2 hours using a microplate reader (EPOCH 2 NS, Agilent Technologies, Santa Clara, CA, USA).

2.7. Evaluation of consumer ratings for the pineapple sticks

Once the optimal preservation treatment was identified, a subsequent experiment was performed with pineapple under ideal conditions to evaluate consumer acceptance of the new product.

For that purpose, a sensory evaluation was conducted using a panel of potential trained consumers ($n=10$) to assess the organoleptic characteristics of pineapple sticks from two different batches: a control batch and an optimized treated batch (*P. acidilactici* CNTA 1059 CFS + Passive MAP). Evaluations were performed on days 0, 8, and 12 of shelf life. Participants assessed key sensory attributes — colour, odour, flavour, and texture — as well as provided an overall product evaluation. A structured, quantitative sensory analysis was employed, using a questionnaire with defined scales: colour was rated on a 0–5 scale (Figure S1), while odour, flavour, and texture were evaluated on a 1–7 intensity scale, ranging from absence to extremely intense. Additionally, a hedonic preference test was conducted using a 1–10 scale, where scores below 5 indicated rejection of the product.

2.8. Statistical analysis

The tables were generated using GraphPad PRISMA software (version 10.3.0). Statistical comparisons were performed assuming normal distribution within each group. For physicochemical parameters, independent-samples *t*-tests were performed to assess significant differences for each variable between 0 and 12 days within each preservation treatment, with significance set at $p \leq 0.05$. One-way ANOVA tests were applied at each time point (for physicochemical parameters), and across all time points (for microbial counts and sensory attributes) to assess significant differences ($p \leq 0.05$) between

treatments. When a significant main effect was detected, pairwise comparisons were subsequently performed using Fisher's least significant difference (LSD) test, and significance was accepted at $p \leq 0.05$.

2.9. Whole genome sequencing (WGS) of the selected LAB strain and post-sequencing analysis

The genome of the selected LAB strain *P. acidilactici* CNTA 1059 was sequenced using the Illumina iSeq100 platform (KIMITEC, Almeria, Spain). Genomic DNA was extracted from morphologically and microscopically confirmed pure cultures using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit. DNA quantity was determined using the Quant-iT™ 1X dsDNA Assay Kit (Thermo Fisher, MA, USA), and quality was evaluated by spectrophotometry (Multiscan Sky, Thermo Fisher) and 0.6% agarose gel electrophoresis. Sequencing libraries were prepared using the Illumina DNA Prep Kit and analyzed with a Bioanalyzer 2100 (Agilent Technologies, CA, USA).

Raw 2×150 bp paired-end reads were quality-checked using FastQC v.0.12.1 (Andrews, 2010) and adapters and low-quality reads were filtered with Trim Galore v.0.6.10. De-novo genome assemblies were generated using shovill v0.9.0 (Seeman, 2017) and the assembly quality (contiguity and completeness) was checked with QUAST v5.2.0 (Gurevich et al., 2013).

The previous taxonomic assignment performed via Sanger sequencing of the 16S rRNA gene was confirmed by analyzing clean reads using Kraken2 v.2.1.3 (Wood et al., 2019), and by submitting the assembled genome to the Type Strain Genome Server (Meier-Kolthoff & Goker, 2019) for whole-genome-based classification, and by comparison of the assembled genome to a closely related reference genome (NCBI RefSeq assembly GCF_013127755.1) via calculation of the Average Nucleotide Identity using FastANI v1.33 (Jain et al., 2018).

Antimicrobial resistance genes were detected using AMRFinderPlus v3.12.8 (Feldgarden et al., 2021), BacAnt (Hua et al., 2021), and ABRicate v1.0.1 (Seeman, 2016) with comprehensive screening across the CARD, MEGARes 2.0, NCBI Bacterial Antimicrobial Resistance Reference Gene Database, ResFinder v4, and ARG-ANNOT databases. To maximize sensitivity, an initial relaxed threshold of 5% identity and coverage was applied to capture all potential hits, followed by manual filtering to retain only high confidence matches with $\geq 80\%$ identity and $\geq 70\%$ coverage (EFSA, 2024). PathogenFinder v1.1 (Cosentino et al., 2013) was used to predict the pathogenic potential of the isolate based on genomic features. Finally, antiSMASH v6.1.1 was used to predict the type and location of secondary metabolite biosynthesis gene clusters within the assembled genomes. In parallel, genome annotation was performed with Prokka v1.14.6 (Seeman, 2014). The resulting annotated sequences were queried against the BAGEL4 database (van Heel et al., 2018) and the APD3 (Antimicrobial Peptide Database) (Wang et al., 2016) using BLAST+ (Camacho et al., 2009) with a minimum identity threshold of 80% to identify genes predicted to encode antimicrobial peptides.

3. Results and discussion

3.1. Identification of the predominant spoilage microbiota associated with fresh-cut pineapple

There was no growth on MRSa and TSA-YE media, but colony growth was observed only on PDA medium, where two morphologically different colonies grew, being responsible for the alteration of the product. They were identified as *Meyerozyma* spp. and *Rhodotorula toruloides*, both of which have been described in the literature as potential spoilage microorganisms of post-harvest pineapples (Manthou et al., 2021).

3.2. Study of the influence of CFS treatment on physicochemical parameters of minimally processed product through preliminary screening

After 12 days of storage, the evaluation of the impact of the addition of the three CFS strains on the physicochemical parameters of the pineapple sticks, through both visual and objective analysis, was essential in selecting and/or discarding potential protective cultures as a preservation option for this RTE product. The findings of this study are detailed below.

The evolution of the headspace concentrations of oxygen and carbon dioxide exhibited no significant differences between the untreated and CFS-treated sticks (data not shown). In all cases, the equilibrium atmosphere was 15% O₂ and 5% CO₂, considered an adequate atmosphere for the commercialization of this type of products. Therefore, there were the factors that proved decisive, and this merely served to ensure that no anomalous changes occurred.

3.2.1. Texture

The results of the firmness of the sticks at start day and after 12 days under refrigeration conditions are presented in Table 1.

At the beginning of the study, there were no significant differences ($p > 0.05$) in the firmness of the sticks between the C1 and C2 control groups and the CFS-treated batches, indicating that immersion in the CFS of lactic bacteria did not affect the sticks' firmness. In the sticks from batches C1, and the treated with the CFS of *L. brevis* CNTA 1374 and *L. plantarum* CNTA 600, no significant differences were observed when comparing the values obtained after 12 days of storage to the initial ones. Similar results were reported by Saravanakumar et al. (2020) in the case of the use of *L. rhamnosus* GG in red and yellow fresh-cut peppers. However, the sticks from C2 batch showed a significant decrease ($p = 0.036$) in firmness, from 11.44 ± 0.70 N to 10.46 ± 0.52 N, aligning with

the findings of Gomez et al. (2020), who reported a decrease in the firmness of pineapple slices packed in MAP conditions and stored at 8°C when the O₂ concentration was between 14% and 17%. The batch treated with the CFS of *P. acidilactici* CNTA 1059 exhibited a significant ($p = 0.011$) increasing trend in firmness over the 12 days of storage, with values of 12.10 ± 0.61 N. Furthermore, the samples treated with this CFS showed significantly higher firmness than those treated with MAP + water, suggesting that *P. acidilactici* CNTA 1059 CFS helped maintain firmness and prevented the softening typically observed in samples packed in MAP alone.

3.2.2. Maturity Index

The effect of the different treatments on the maturity index of the pineapple sticks is detailed in Table 1. No significant differences were observed between the batches at the initial moment. Both control batches and those packed with MAP and treated with *L. brevis* CFS CNTA 1374 had values of 1.37 ± 0.24 , while the batches packed in MAP and treated with the CFS of *P. acidilactici* CNTA 1059 or *L. plantarum* CNTA 600 had values of 1.29 ± 0.11 . Over the 12-day refrigerated storage period, both the controls and CFS-treated sticks showed an increase in maturity index, likely due to ripening or water loss (Martínez-Castellanos et al., 2011; Temiz & Özdemir, 2021). Batches with a lower initial maturity index exhibited a significant increase ($p < 0.001$), with values of 1.93 ± 0.11 (*P. acidilactici* CNTA 1059) and 1.92 ± 0.15 (*L. plantarum* CNTA 600), which were also significantly higher than those observed in the other batches at this time point.

3.2.3. Colour

The colour evolution (ΔE) and the % of browning during the storage varied depending on the combined treatment applied. Table 1 summarizes the ΔE results and the browning percentage obtained with the digital image analyzer after 12 days of storage. These results are presented for C1 and C2 and the sticks treated with different CFSs

packed in passive MAP. Additionally, Figure S2 shows, shown as an example, representative images overlaid with CellProfiler segmentation outputs, with red areas identified as browning on the pineapple sticks.

The initial colour of the sticks from different batches was not affected (data not shown) by immersion in the CFS of lactic acid bacteria. These results aligned with (Russo et al., 2014), who did not detect significant differences when immersing pineapple pieces in suspensions of *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum*.

The ΔE values of the control sticks packaged in air (C1) were very similar to those of the control sticks packed under passive MAP (C2), with values of 12.75 ± 1.25 and 12.22 ± 0.49 , respectively. Similarly, the CellProfiler recorded the percentage of browning for each batch as 32.63% and 39.34%, respectively.

However, the sticks immersed in the other CFSs showed a markedly different colour evolution compared to the control and to each other. The CFS of *L. plantarum* CNTA 600 altered the colour of the sticks, resulting in a higher colour difference ($\Delta E = 14.20 \pm 2.40$) and browning percentage (46.42%) compared to other treatments. This effect was not observed in other studies where *L. plantarum* was used as a probiotic ingredient in edible coatings for various ready-to-eat fruits and vegetables, such as apple slices (Wong et al., 2021) or cherry tomatoes (Álvarez et al., 2021).

For the other two lactic acid bacteria, *P. acidilactici* CNTA 1059 and *L. brevis* CNTA 1374, showed the ability to inhibit the browning of pineapples compared to the controls and CFS *L. plantarum*-treated sticks, as indicated by lower ΔE values of 12.11 ± 0.49 and 11.14 ± 0.75 , respectively. The results obtained by the CellProfiler with the sticks treated with the CFS of *L. plantarum* CNTA 600 and MAP packaging, was not aligned with the results described by Martínez-Castellanos et al. (2011), who described the use of *L. plantarum* as an option to protect the colour of the lichi fruit. The lesser change in

initial colour was also reflected in the CellProfiler analysis, where the browning percentages were 9.46% for the sticks treated with *P. acidilactici* and 11.54% for those treated with *L. brevis* CNTA 1374 CFS, what confirmed the information detailed by Agriopoulou et al. (2020) describing bacteriocins as potential anti-browning agents.

After evaluating the impact of the 3 CFSs on the colour of the pineapple sticks, it can be concluded that the combined treatment of passive MAP packaging and immersion in *L. plantarum* CNTA 600 CFS it was not suitable and was discarded for application because it negatively affected the characteristic colour of the product. For this reason, the following sections will only discuss the CFS of the two remaining LAB strains.

3.3. Evaluation of the antimicrobial potential of the CFSs to select the most effective treatment

Monitoring the wild microbial evolution of the pineapple sticks was crucial for selecting the optimal combination treatment to extend the product's shelf life. Consequently, the yeasts responsible for the spoilage of the sticks were tracked over the 12 days of storage.

The results of the microbial concentration for the different batches can be seen in Table 2. Initial values ranged from around 1 to 3 log CFU/g, depending on the initial batch, highlighting the heterogeneity of the product (Gomez et al., 2020). Microbial growth remained stable for the first days of the study, showing similar growth across all samples up to 8 days (not shown), consistent with the findings reported by Russo et al. (2014). However, between days 8 and 12, the evolution of the yeasts varied depending on the batch. At day 12, the sticks from control batches C1 and C2 showed the highest yeast concentrations (8.94 ± 0.54 and 8.02 ± 0.24 log CFU/g, respectively).

For the CFS-treated sticks, a reduction in the growth rate was observed, attributed to the synergistic effect of both the CFS and the MAP packaging, resulting in an extension

of the product's shelf life (Hashemi & Jafarpour, 2021). These exhibited counts of around 6 log CFU/g. The batch treated with *L. brevis* CNTA 1374 CFS showed a growth of 4.31 ± 1.94 log CFU/g from time 0 to 12 days. This species is known for its antifungal activity against *Fusarium verticillioides* (Somashekaraiah et al., 2021).

The combined treatment of *P. acidilactici* CNTA 1059 CFS and MAP demonstrated the lowest microbial growth, with an overall increase of only 2.93 ± 0.15 log CFU/g from the initial time point. This effect is likely due to the antifungal capacity of *P. acidilactici* strains as highlighted by Das et al. (2020) and Fugaban et al. (2022). This finding suggests that the CFS reduced the growth of spoilage microorganisms by acting as an additional protective barrier, in line with the findings of Tenea et al. (2020), who reported microbial reduction after the application of an edible coating with LAB on pineapple slices.

Therefore, considering the physicochemical and microbial characteristics, it was determined that the optimal combined treatment for this product involves passive MAP packaging and immersion in the CFS of the lactic acid bacteria *P. acidilactici* CNTA 1059. Figure 1 shows the flowchart used to obtain the CFS-treated and control batches of pineapple sticks after completing the optimization process.

3.4. Validation of the selected treatment through testing under abusive temperatures and a consumer-based sensory evaluation

3.4.1. Microbial analysis under abusive temperatures

Given the promising results obtained with the *P. acidilactici* CNTA 1059 CFS from physicochemical and microbial results, a follow-up microbial analysis was conducted comparing the air + water control treatment and the combined MAP + *P. acidilactici* CNTA 1059 CFS treatment under temperature abuse conditions (10 days/ 10 °C). As shown in Table 3, yeast concentrations in the control samples (air + water) reached 8.26 ± 0.50 log CFU/g after 5 days and 9.10 ± 0.18 log CFU/g after 10 days at 10 °C.

Compared to the control samples, those treated with *P. acidilactici* CFS and packed under MAP exhibited significantly ($p < 0.001$) lower yeast levels after 5 days (4.32 ± 0.36 log CFU/g) and 8.80 ± 0.51 log CFU/g after 10 days. Thus, the combined treatment effectively extended the shelf life by 50% of the pineapple sticks despite potential breaks in the cold chain.

3.4.2. Evaluation of consumer ratings

Table 4 presents the evolution of selected sensory parameters according to the consumers of fresh-cut pineapple without any preservation treatment and with the selected combined treatment: CFS of *P. acidilactici* CNTA 1059 packed in MAP. The results from the global assessment indicated that the control pineapple sticks became inedible by day 8 of storage, whereas the treated samples remained acceptable up to day 12. Consequently, the evaluation of flavour and texture parameters in the control samples was discontinued after 8 days. This earlier inedibility of control pineapples compared to treated ones is consistent with findings by Basumatary et al. (2022), who treated the pineapples with edible coatings containing lactic acid bacteria.

Over the storage period, the control batch of pineapple sticks significantly ($p = 0.001$) darkened in colour, while the CFS-treated batch maintained its typical colour (Figure S3). This issue aligned with the findings of De Simone et al. (2024) on the beneficial influence of edible coatings with probiotics on the colour of strawberries, as well as with Rößle et al. (2010) regarding the colour evolution of apple slices in the presence of the probiotic *L. rhamnosus*. This differs from the results reported by Mantilla et al. (2013), who noted colour maintenance in products stored at 4 °C for 13 days. Initially, testers noted significant differences in the odour and flavour profiles ($p < 0.001$ and $p = 0.017$, respectively) of the two batches, being higher for the CFS-treated one. The control batch developed musty and rotten smells over time, consistent with Montero-

Calderón et al. (2008), whereas the CFS-treated batch retained a fresh pineapple odour and high flavour scores throughout the 12 days. This suggests that *P. acidilactici* CNTA 1059 CFS compounds did not contribute with off-flavours in the sticks, aligning with Russo et al. (2014) for probiotic-treated apple slices. At the initial time point both batches showed similar texture, but the CFS-treated sticks maintained their texture over 12 days, while the control batch became softer, as observed but not formally tested by Mantilla et al. (2013). Finally, in terms of taster acceptance, the treated sticks consistently received higher scores throughout the storage period compared to the untreated ones. Initially, the first ones scored nearly one point higher (7.60 ± 0.39), with a slight decrease to 7.13 ± 0.48 after 12 days. In contrast, the control batch was rejected (score < 5) from day 8 onwards due to microbial growth on the surface.

In summary, the combined treatment with *P. acidilactici* CNTA 1059 CFS and Passiver MAP effectively preserved the sensory quality and extended the acceptability of fresh-cut pineapple sticks, maintaining colour, odour, texture, and overall acceptance for up to 12 days, in contrast to the rapid deterioration observed in the untreated control. Importantly, the efficacy of the treatment was also confirmed under temperature abuse conditions, simulating potential breaks in the cold chain during distribution. All these findings validate the designed preservation strategy and support its robustness and potential for commercial application.

3.5. Exploration of the underlying mechanisms responsible for the antimicrobial activity of the selected CFS

In order to explore the possible mechanism by which the CFS exhibits antimicrobial activity against the microorganisms of the pineapple sticks, a sensitivity assay, WGS of the producing strain, and protein characterization of the CFS were performed. Details of

the procedure for the protein characterization (SDS-PAGE, in-gel digestion, zymography and LC-MS/MS identification) are provided in the Supplemental Material and Methods.

3.5.1. In vitro antimicrobial activity of the CFS

A MIC determination assay was performed using increasing concentrations of the *P. acidilactici* CNTA 1059 CFS to evaluate its in vitro inhibitory activity against the two yeasts identified. As shown in Figure 2, a dose-dependent inhibitory effect was observed, with 5% CFS inhibiting the growth of *R. toruloides*.

3.5.2. Whole genome sequencing

Whole Genome Sequencing was performed to confirm the taxonomic identity of the isolate, assess the absence of acquired antibiotic resistance and pathogenicity determinants, and detect biosynthetic cluster genes related to the production of bacteriocins or other secondary metabolites. This genomic evaluation aligns with EFSA recommendations (EFSA, 2005, 2024) for the use of microbial strains in food applications.

WGS-based taxonomic classification of the isolate was conducted using Kraken2, which identified it as *Pediococcus acidilactici*, a species included in the EFSA Qualified Presumption of Safety (QPS) list (EFSA BIOHAZ Panel, 2024). Taxonomic assignment was further validated using the Type Strain Genome Server (TYGS), which confirmed the isolate's affiliation with a well-supported *Pediococcus acidilactici* clade, showing digital DNA–DNA hybridization values ranging from 75% to 89% when compared with *P. acidilactici* type strains. In addition, FastANI analysis revealed an average nucleotide identity (ANI) of 98.56% with the reference genome of *P. acidilactici* strain PMC65, exceeding the species delineation threshold of 95%, therefore supporting its classification within the same species.

Regarding the search for antibiotic resistance genes, none of the low-confidence hits (Table S2) exceeded the high-confidence filtering threshold ($\geq 80\%$ identity, $\geq 70\%$ coverage). These results, together with the previously performed phenotypic MIC testing (Garin-Murguialday et al., 2024), show that *P. acidilactici* CNTA 1059 does not appear to carry acquired antibiotic resistance determinants of concern. Furthermore, the strain was deemed not pathogenic to humans after evaluation with PathogenFinder (prediction score of -24.564).

Functional genome mining using antiSMASH v6.1.1 only revealed a bacteriocin biosynthetic gene cluster sharing 80% similarity with the known coagulin cluster. Subsequent BLAST analysis against the BAGEL4 and APD3 databases identified this cluster as consistent with pediocin PA-1 biosynthesis, and key genes associated with the pediocin operon (*pedA*, *pedC*, and *pedD*)—were detected with 100% identity and coverage (Table S3).

3.5.3. Protein characterization of the CFS by SDS-PAGE

Figure S4 shows the results of the SDS-PAGE assay conducted with the CFS of *P. acidilactici* CNTA 1059. Various bands could be observed in the CFS; and a band was detected around 5 kDa, consistent with the expected size range of pediocin PA-1 (4.6 kDa), despite the resolution limitations of the gel preventing precise identification. This band would correspond to the size of pediocin PA-1, with a molecular weight of 4.6 kDa (Zhu et al., 2022).

3.5.4. LC-MS/MS identification

Mass spectrometry analysis of the 4.6 kDa band in the CFS enabled the detection of protein fragments consistent with known bacteriocins produced by *Pediococcus acidilactici*. Specifically, peptides corresponding to pediocin PA-1 and its associated

biosynthetic components (PedA, PedC, and PedD) were identified in the CFS, confirming the presence of bacteriocin-related proteins (Table S4).

3.5.5. Hypothetical mechanistic antimicrobial effect of the CFS

The findings reported by Garín-Murguialday et al. (2024) demonstrated that *Pediococcus acidilactici* CNTA 1059 produces several organic acids, including lactic acid, acetic acid, and notably phenyllactic acid, a well-known antifungal metabolite (Fugaban et al., 2022). In vitro assays confirmed that a 5% concentration of the CFS effectively inhibited the growth of *Rhodotorula toruloides*, one of the yeasts detected in fresh-cut pineapple samples. However, no other proteinaceous compounds with established antifungal activity were identified in the present study, highlighting the need for further studies to fully demonstrate the antifungal mechanisms involved.

In parallel, genome mining revealed a pediocin PA-1 biosynthetic gene cluster, suggesting the strain's potential to produce bacteriocins. This was further supported by mass spectrometry, which confirmed the presence of pediocin PA-1-related peptides in the CFS. Additionally, a zymography assay (an electrophoresis technique that detects antimicrobial activity directly within a gel matrix) was performed using *Listeria monocytogenes* as an indicator strain, revealing an active antimicrobial band matching the expected molecular weight of pediocin PA-1 (Figure S5) and further corroborating its production by *P. acidilactici* CNTA 1059.

Both acidification of the environment and production of pediocins are characteristic of *P. acidilactici*, which has been studied for its probiotic potential and its impact on the gut microbiota (Mokrani M., 2024). Organic acids act by disrupting pH homeostasis and impairing fungal metabolism, while pediocin specifically targets Gram-positive bacteria by forming pores in their membranes, leading to rapid cell death (Zhu et al., 2022). This complementary mode of action is likely to result in a synergistic antimicrobial effect, not

only broadening the spectrum of microbial inhibition but also potentially reducing the likelihood of resistance development.

Although pediocin itself is not currently approved as a direct food additive in Europe (Comission Regulation (EU) 1333/2008) or USA (FDA, 2024), its natural production by food-grade LAB strains offers a promising route for its indirect use via protective cultures.

To bring the product into the market, further tests are planned following this study. These assays will focus on optimizing and producing a medium suitable for human consumption that promotes the production of phenyllactic acid and bacteriocins. The main objective is to reduce the concentration of the CFS of *P. acidilactici* CNTA 1059 in the final product while maintaining the same organoleptic characteristics and achieving or improving the shelf-life results obtained with the 5 % of CFS.

4. CONCLUSIONS

The combined application of the immersion in the CFS from *P. acidilactici* CNTA 1059 and Passive MAP effectively reduced the proliferation of *Meyerozyma* spp. and *Rhodotorula toruloides* yeasts naturally present in the pineapple sticks after 12 days of storage at refrigeration temperatures (4 °C for 4 days and 8 °C for 8 days) compared to the control batch. Moreover, the synergistic effect of these two combined techniques preserved the physicochemical properties of the sticks extending their shelf life by up to 12 days without compromising their sensory quality.

The antifungal activity of the CFS of *P. acidilactici* CNTA 1059 was demonstrated and attributed primarily to the production of organic acids. In addition, the production of pediocin, which has also shown to be an effective inhibitor of the enzymatic browning reaction, by this strain was confirmed through genome analysis of the bacteria and

molecular characterization of the CFS. Importantly, in compliance with EFSA's regulatory framework (EFSA, 2024), the original strain harboured was recognized as belonging to the QPS list (EFSA BIOHAZ Panel, 2024), had no transmissible antibiotic resistance genes, and was deemed as non-pathogenic, further confirming previous phenotypic assays that supported the non-toxigenic characteristics of this microorganism (Garin-Murguialday et al., 2024).

P. acidilactici CNTA 1059 emerges as a promising candidate for replacing E-number preservatives in minimally processed foods (like fresh-cut fruits) due to its natural origin, proven efficacy, and potent antimicrobial and antibrowning activity. These attributes strongly support its potential integration into biopreservation strategies designed to meet clean-label demands while extending shelf-life and preserving the sensory properties of food products.

CRedit authorship contribution statement

Nerea Garin-Murguialday: Investigation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing. Francisco Márquez-Urbano: Investigation, Visualization. Rafael Pagán: Funding acquisition, Conceptualization, Writing - Review & Editing. Raquel Virto: Funding acquisition, Conceptualization, Supervision. Laura Espina: Formal analysis, Data curation, Writing - Review & Editing. Esther Arias: Conceptualization, Formal analysis, Supervision, Writing - Review & Editing.

Funding sources

This research was funded by Gobierno de Navarra, funding allocation G20001-G2100-4701- 467300: Contratación personal investigador y tecnológico, doctorados industriales—project BacBiop (0011-1408-2022-000003). This research was also funded

by MCIN/AEI/10.13039/501100011033, Grant PID2021-123404NB-I00, by “ERDF A way of making Europe” and by the Government of Aragón (grant Grupo AESA).

Declaration of competing interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Acknowledgments

The University, Innovation and Digital Transformation Department, R&D&I Service of the Government of Navarra (Spain) provided N. Garin-Murguialdy with a grant to carry out this research.

Appendix A. Supplementary material

Supplementary data to this article can be found online at [insert link].

Data Availability Statement

The raw sequencing reads were deposited in the European Nucleotide Archive (accession number of the project: PRJEB74905, sample SAMEA118005737). The rest of the raw data is available at the provided link: <https://doi.org/10.5281/zenodo.15428149>.

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Figure captions

Figure 1. Optimized flowchart for the production of pineapple sticks treated with cell-free supernatant (CFS) and their respective control.

Figure 2. Absorbance measurements of *Rhodotorula toruloides* in the presence of 0% (●), 0.15% (■), 2.5% (▲), and 5% (◇) (v/v) of CFS. Data represent the mean $\pm$ standard deviation of three biological replicates.

Tables

Table 1. Evolution of physicochemical parameters (firmness, maturity index, colour and browning) of CFS *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600 and *L. brevis* CNTA 1374-treated pineapple sticks during 12 days at 4°C. For each parameter, an asterisk indicates a significant difference ($p \leq 0.05$) in firmness within each treatment between 0 and 12 days (*t*-test), while superscript letters indicate significant differences between treatments at day 12 (based on one-way ANOVA followed by Fisher's LSD test); means not sharing a letter differ significantly ($p \leq 0.05$). Data expressed as mean $\pm$ standard deviation of 5 independent replicates.

Preservation treatment	Time (days)	Firmness (N)	Maturity index	Colour (ΔE)	Browning (%)
C1 (air + water)	0	11.44 $\pm$ 0.70	1.37 $\pm$ 0.24	-	-
	12	11.24 ^{abc} $\pm$ 1.29	1.64 ^a $\pm$ 0.16	12.75 ^{abc} $\pm$ 1.25	32.63 ^a $\pm$ 7.65
C2 (passive MAP + water)	0	11.44 $\pm$ 0.70	1.37 $\pm$ 0.24	-	-
	12	10.46 ^{b*} $\pm$ 0.52	1.48 ^b $\pm$ 0.05	12.22 ^{abc} $\pm$ 0.95	39.34 ^{ac} $\pm$ 8.16
passive MAP + CFS <i>P. acidilactici</i> CNTA 1059	0	11.03 $\pm$ 0.40	1.29 $\pm$ 0.11	-	-
	12	12.10 ^{c*} $\pm$ 0.61	1.93 ^{c*} $\pm$ 0.11	12.11 ^{abc} $\pm$ 0.49	9.46 ^b $\pm$ 5.55
passive MAP + CFS <i>L. brevis</i> CNTA 1374	0	11.44 $\pm$ 0.70	1.37 $\pm$ 0.24	-	-
	12	11.41 ^{abc} $\pm$ 0.82	1.43 ^b $\pm$ 0.12	11.14 ^b $\pm$ 0.75	11.54 ^b $\pm$ 4.19
passive MAP + CFS <i>L. plantarum</i> CNTA 600	0	11.03 $\pm$ 0.40	1.29 $\pm$ 0.11	-	-
	12	11.80 ^{ac} $\pm$ 0.83	1.92 ^{c*} $\pm$ 0.15	14.20 ^c $\pm$ 2.40	46.42 ^c $\pm$ 6.64

Table 2. Total yeast counts (log CFU/g) of the different stick batches under refrigerated storage (4 days at 4 °C followed by 8 days at 8 °C). Superscript letters indicate significant differences between treatments (based on one-way ANOVA followed by Fisher's LSD test); means not sharing a letter differ significantly ($p \leq 0.05$). Data expressed as mean $\pm$ standard deviation of 5 independent replicates.

Preservation treatment	Time (days)	
	0	12
C1 (air + water)	2.70 ^a $\pm$ 0.02	8.94 ^d $\pm$ 0.54
C2 (passive MAP + water)	3.12 ^b $\pm$ 0.34	8.02 ^e $\pm$ 0.24
passive MAP + CFS <i>P. acidilactici</i> CNTA 1059	3.14 ^b $\pm$ 0.20	6.07 ^f $\pm$ 0.10
passive MAP + CFS <i>L. brevis</i> CNTA 1374	1.61 ^c $\pm$ 0.08	6.00 ^f $\pm$ 0.20

Table 3. Total yeast counts (log CFU/g) of the different stick batches under abusive temperatures (10 °C). Superscript letters indicate significant differences between treatments (based on one-way ANOVA followed by Fisher's LSD test); means not sharing a letter differ significantly ($p \leq 0.05$). Data expressed as mean $\pm$ standard deviation of 5 independent replicates.

Preservation treatment	Time (days)		
	0	5	10
C1 (air + water)	3.72 ^a $\pm$ 0.60	8.26 ^b $\pm$ 0.50	9.10 ^b $\pm$ 0.18
passive MAP + CFS <i>P. acidilactici</i> CNTA 1059	3.92 ^{ac} $\pm$ 0.33	4.32 ^c $\pm$ 0.36	8.80 ^b $\pm$ 0.51

Table 4. Sensory analysis of pineapple sticks of control batch and combined treated batch of MAP + CFS *P. acidilactici* CNTA 1059 at 12 days. Controls were sticks without treatment with any CFS stored at 4 °C. For each attribute, statistically significant differences ($p \leq 0.05$) were determined using one-way ANOVA followed by Fisher's LSD test, and are indicated by different superscript letters. 'u.t.' = untested. Data expressed as mean $\pm$ standard deviation.

Time (days)	Control*	<i>P. acidilactici</i> CNTA 1059 CFS + passive MAP
Colour		
0	2.85 ^a $\pm$ 0.46	3.80 ^c $\pm$ 0.59
8	3.38 ^b $\pm$ 0.77	3.87 ^c $\pm$ 0.21
12	3.36 ^b $\pm$ 0.52	3.82 ^c $\pm$ 0.65
Odour		
0	2.31 ^a $\pm$ 0.37	2.92 ^c $\pm$ 0.19
8	2.16 ^a $\pm$ 0.34	3.30 ^d $\pm$ 0.39
12	1.78 ^b $\pm$ 0.14	3.37 ^d $\pm$ 0.18
Flavour		
0	3.78 ^a $\pm$ 0.62	4.53 ^b $\pm$ 0.28
8	u.t.	4.10 ^{ab} $\pm$ 0.41
12	u.t.	4.01 ^{ab} $\pm$ 0.51
Texture		
0	2.42 ^b $\pm$ 0.37	2.75 ^{a,b} $\pm$ 0.46
8	u.t.	3.28 ^a $\pm$ 0.50
12	u.t.	2.96 ^{a,b} $\pm$ 0.25
Global assessment		
0	6.81 ^a $\pm$ 1.04	7.60 ^b $\pm$ 0.39
8	<5	7.40 ^b $\pm$ 0.11
12	<5	7.13 ^{ab} $\pm$ 0.48

Figure 1. Optimized flowchart for the production of pineapple sticks treated with cell-903 free supernatant (CFS) and their respective control.

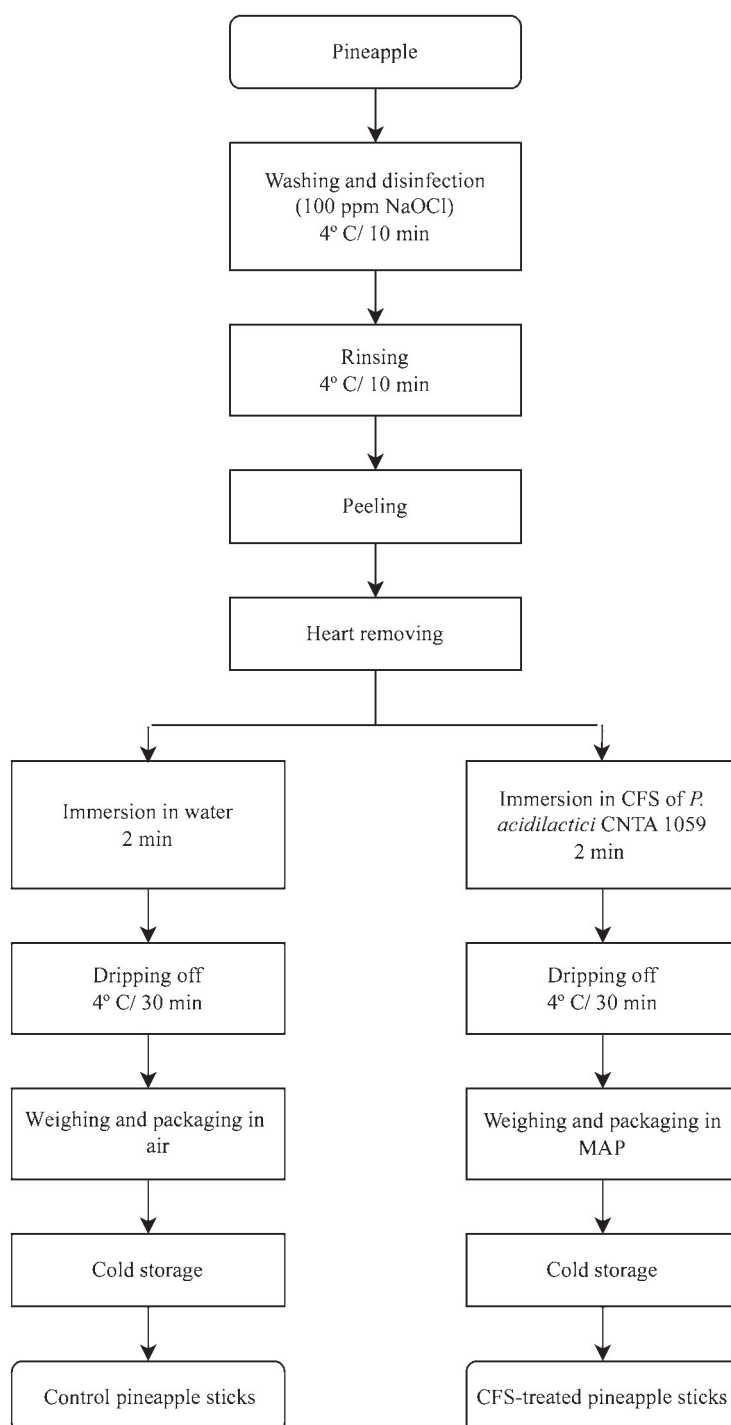
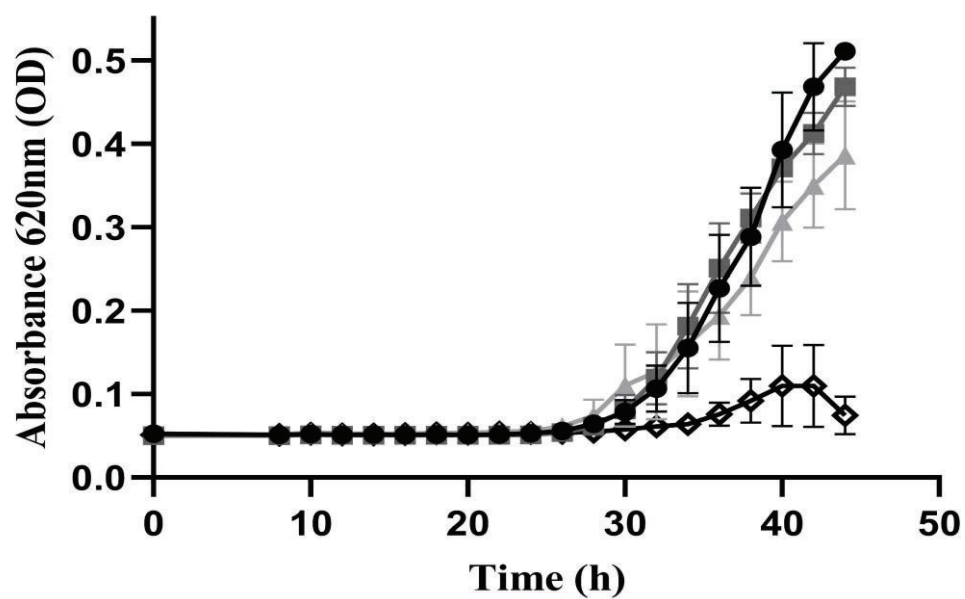


Figure 2. Absorbance measurements of *Rhodotorula toruloides* in the presence of 0% 905 (●), 0.15% (■), 2.5% (▲), and 5% (◇) (v/v) of CFS. Data represent the mean $\pm$ standard deviation of three biological replicates.



Supplemental material for:

Quality preservation of pineapple sticks using the cell-free supernatant of protective cultures

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Supplemental Material and methods

Protein characterization of the selected CFS by SDS-PAGE and in-gel digestion

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the protein profile of the strain and estimate the molecular weight of the antimicrobial proteinaceous compounds present in the CFS.

To obtain partially purified bacteriocin extracts, a 1 L culture of the protective strain were grown for 24 h, centrifuged, and filtered to produce the CFS. Ammonium sulfate (80% saturation; Merck) was added to each CFS, followed by gentle agitation at 10 °C overnight. The precipitate was collected by centrifugation at $10,000 \times g$ for 10 min (Eppendorf 5910 Ri, Hamburg, Germany), and the pellet was resuspended in 10 mL of 0.2 M phosphate-buffered saline (PBS; OXOID, Madrid, Spain). Protein extracts were then dialyzed overnight at 8 °C against distilled water using 2 kDa molecular weight cut-off (MWCO) dialysis membranes (Thermo Fisher). The next day, dialyzed proteins were recovered for analysis.

For electrophoresis, a 16% acrylamide gel was prepared, and samples were separated using a modified Tris/Tricine SDS-PAGE protocol (Schagger, 2006). Dithiothreitol (DTT; PanReac, Barcelona, Spain) was added to the dialyzed samples along with sodium dodecyl sulfate (SDS; PanReac), and the mixture was incubated at 37 °C for 60 min to ensure denaturation. Each sample (40 µL) and two molecular weight markers (PageRuler™ Prestained Protein Ladder, 10–180 kDa, Ref. 26616; and Spectra™ Multicolor Low Range Protein Ladder, Ref. 26628; Thermo Fisher) were loaded onto the gel and run at 60 V for 5 h.

After electrophoresis, gels were fixed (50% ethanol, 10% glacial acetic acid, 40% distilled water) for 30 min and stained overnight with Coomassie Colloidal solution (Dybala and Metzger, 2009). Gels were then destained with 30% ethanol for 4 hours and imaged using ImageJ (BIO-RAD, Berkeley, CA, USA) for molecular weight comparison and band selection.

Candidate bands corresponding to the expected molecular weight of pediocin PA-1 were excised from the SDS-PAGE gel and subjected to in-gel digestion based on the protocol by Shevchenko et al. (2006), with minor modifications. Coomassie stain was removed by incubating gel pieces in 50% acetonitrile (ACN; Sigma-Aldrich) and 50 mM ammonium bicarbonate (ABC; Thermo Fisher) at 40 °C. Bands were dehydrated in 100% ACN, reduced with 10 mM DTT, and alkylated using 55 mM iodoacetamide (IAA; Thermo Fisher).

After final dehydration, overnight trypsin digestion was carried out at 37 °C. Peptides were extracted using 2% ACN in 0.5% formic acid (FA; Thermo Fisher), and collected in LoBind Eppendorf tubes. Then, 30 µL of 100% ACN was added to the band and incubated for 5 min. This step was repeated three times until the gel appeared white. The resulting peptide mixture was dried and submitted for LC-MS/MS analysis.

Zymography

To identify proteins exhibiting antimicrobial activity and determine their molecular weights, zymography was performed in parallel to the acrylamide gel, as described by Wilkesman and Kurz (2022). The zymogram gel was also run for 5 hours at 60 V, fixed as described above, and left in sterile distilled water overnight. The following day, the gel lane containing the CFS from *Pediococcus acidilactici* CNTA 1059 was excised and placed on a Petri dish (VWR, Radnor, PA, USA). The gel slice was then overlaid with *Listeria monocytogenes* CECT 7467, the indicator strain that showed the highest susceptibility to this CFS as previously determined (Garin-Murguialday et al., 2024). The indicator strain was prepared at a concentration of 10^6 CFU/mL in 0.8% agar. The plate was incubated at 8 °C for 6 h, followed by aerobic incubation at 37 °C for 17 h. Zones of

inhibition were compared to protein bands on the SDS-PAGE gel to correlate antimicrobial activity with molecular weight.

LC-MS/MS identification

For LC-MS analysis, lyophilized peptides were reconstituted with 0.1% formic acid and quantified using a NanoDrop spectrophotometer (ThermoFisher). LC-MS/MS was performed with an Evosep One (Evosep Biosystems, Odense, Denmark) coupled to an Exploris Orbitrap 480 mass spectrometer (ThermoFisher Scientific), loading 50 ng of peptides in an Evotip. Peptide resolution was performed using a C18 Aurora Ultimate column (75 μm x 15 cm, particle size of 1.7 μm) (IonOpticks, Melbourne, Australia) with the WhisperZoom (Evosep Biosystems) 20 samples per day method. Data were acquired in a data-dependent mode with specific settings for MS and MS/MS scans. Peptides were fragmented using HCD collision energy of 30%. Data analysis was conducted using Spectromine (v 4.5.240625.52329) (Biognosys, Zurich, Switzerland), with dynamic MS1/MS2 calibration and specific modifications. The enzyme was set to trypsin, and the FDR filter was set to 1%. Data from this sample was searched against a fasta file containing protein sequences from *P. acidilactici* CNTA 1059.

Supplemental Figures

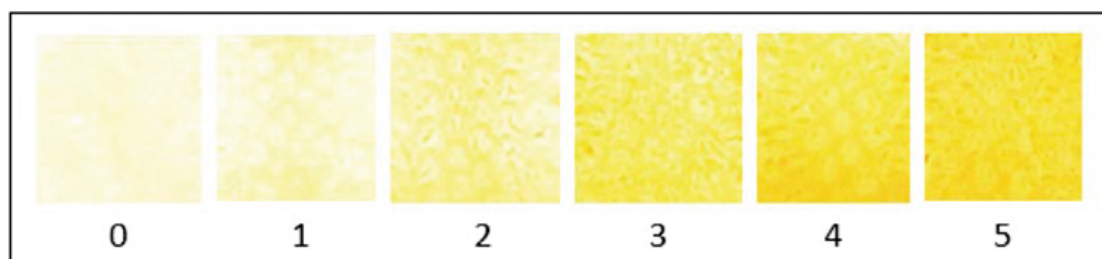


Figure S1. Chart of yellow colours used to determine the colour of the sticks during tasting.

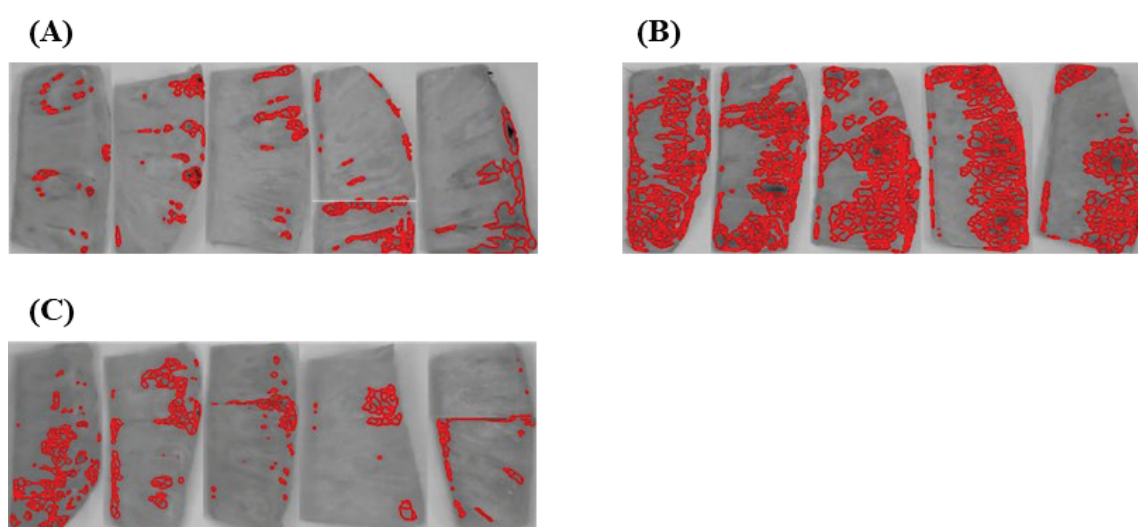


Figure S2. The effect in pineapple sticks detected by CellProfiler of the combination between the CFS of *P. acidilactici* CNTA 1059 and MAP packaging (A), *L. plantarum* CNTA 600 and MAP packaging (B), and *L. brevis* CNTA 1374 and MAP packaging (C) after 4 days at 4°C and 8 days at 8°C (5 replicates) after the image analysis by CellProfiler.



Figure S3. Photographs of pineapple sticks presented and tasted by consumers after 4 days at 4°C and 8 days at 8°C. Untreated control stick (left) and stick treated with the combination of the CFS of *P. acidilactici* CNTA 1059 and MAP (right).

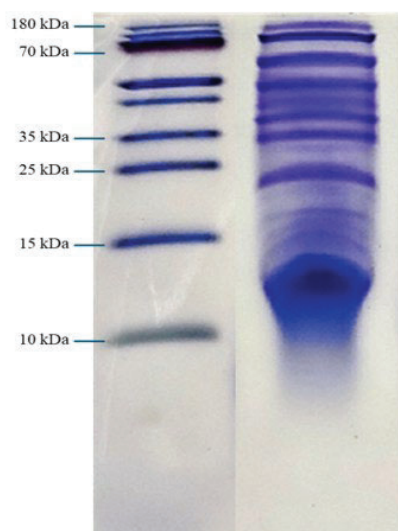


Figure S4. Tricine SDS-PAGE: molecular marker PageRuler™ (10 to 180 kDa) (left) and peptide band of *P. acidilactici* CNTA 1059 (right).

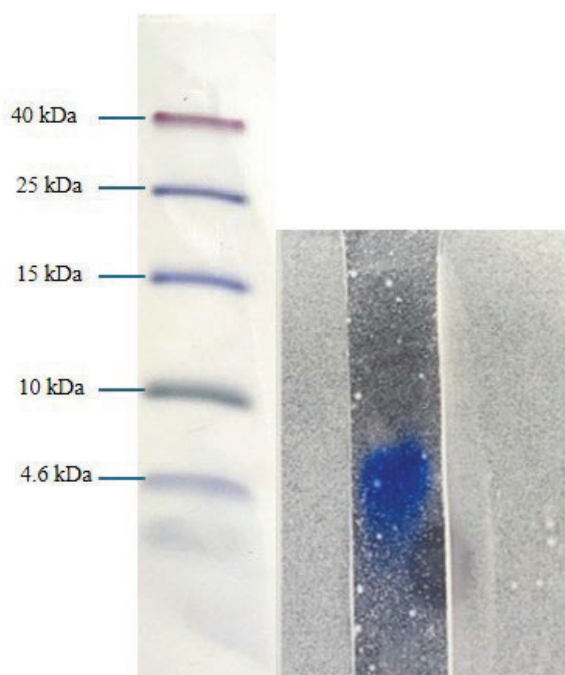


Figure S5. Results from zymography. Molecular marker Protein Marker, Low Range (1.7-42 kDa) (left), and peptide band of *P. acidilactici* CNTA 1059, where the gel was covered with viable cells of *L. monocytogenes* CECT 7467 (10⁶ CFU/mL) on TSA-YE agar at 37 °C for 24 hours (right).

Supplemental Tables

Table S1. Growth conditions used for the isolation and identification of pineapple spoilage microorganisms.

Microorganism	Growth conditions			
	Medium	Temperature (°C)	Oxygen conditions	Time (h)
Lactic Acid Bacteria	MRSa*	30	Anaerobic	48
Lactic Acid Bacteria	MRSa*	37	Anaerobic	48
Aerobic mesophiles	TSA-YE**	37	Aerobic	24
Yeasts and moulds	PDA***	25	Aerobic	48 - 72

*MRSa: Man, Rogosa and Sharpe Agar (Merck GmbH, Darmstadt, Germany)

**TSA-YE: Tryptic Soy Agar (Scharlau, Barcelona, Spain)

***PDA: Potato Dextrose Agar (Scharlau, Barcelona, Spain)

Table S2. Antibiotic resistance genes identified in the *Pediococcus acidilactici* CNTA 1059 genome after applying all tools to the assembled genome with relaxed settings ($\geq 5\%$ coverage and identity), indicating the tool and database used, the genomic coordinates, and the percentages of coverage and identity.

Tool	Database	Gene	%Coverage	%Identity
ABRicate	MEGARES	<i>efrB</i>	51.01	66.31
BacAnt	resDB	<i>chrR_I</i>	28.88	69.14
BacAnt	resDB	<i>lsa(A)</i>	10.55	69.62
BacAnt	resDB	<i>qacH</i>	34.92	71.21
BacAnt	resDB	<i>vat(A)</i>	15.15	75.00
BacAnt	resDB	<i>vanR-C</i>	18.25	72.44
BacAnt	resDB	<i>bpsX</i>	15.77	72.14
BacAnt	resDB	<i>bpsX</i>	23.09	78.54
BacAnt	resDB	<i>cesD</i>	8.79	76.62
BacAnt	resDB	<i>copB_Eh</i>	10.28	68.26
BacAnt	resDB	<i>copB_Eh</i>	21.85	67.48
BacAnt	resDB	<i>copB_Eh</i>	19.17	67.37
BacAnt	resDB	<i>tetA(58)</i>	21.41	70.14

Table S3. BLASTp hits of Prokka-annotated genes from the *Pediococcus acidilactici* CNTA 1059 genome against bacteriocin-related proteins from the BAGEL4 database.

Database	Matched gene ID	Matched entry	%Coverage	%Identity
BAGEL4	UniProt P36496	PedA	100.00	100.00
BAGEL4	UniProt PEDC_PEDAC	PedC	100.00	100.00
BAGEL4	UniProt PEDD_PEDAC	PedD	100.00	100.00
APD3	AP00634	Pediocin PA-1	100.00	100.00

Table S4. Protein fragments identified by mass spectrometry analysis of the ~4.6 kDa band from the CFS of *Pediococcus acidilactici* CNTA 1059. In bold are the peptides corresponding to pediocin PA-1.

Entry	Protein names	Gene Names	Length	MW (Da)	Gene Ontology (biological process)	Gene Ontology (molecular function)	Gene Ontology (GO)	Gene Ontology IDs
A0AAW8YF R5	YhgE/Pip family protein	R0G89_04055	1065	111109				
E0NH38	Methyl-accepting chemotaxis protein signaling domain protein	HMPREF0623_1 235	1065	111038		ABC-type transporter activity [GO:0140359]	plasma membrane [GO:0005886]; ABC-type transporter activity [GO:0140359]	GO:0005886; GO:0140359
A0AAW8YJ 68	AAA family ATPase	R0G89_10525	735	81780				
E0NHK4	ATPase family associated with various cellular activities (AAA)	clpE HMPREF0623_1 527	735	81779	cellular response to heat [GO:0034605]	ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]	cytoplasm [GO:0005737]; ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]; cellular response to heat [GO:0034605]	GO:0005524; GO:0005737; GO:0005524; GO:0016887; GO:0034605
A0AAW8YT 86	AAA family ATPase	R0H03_09975	735	81741				
A0AAW8YP A3	Elongation factor G	fusA R0H03_05455	697	77041				
E0NFF0	Elongation factor G (EF-G)	fusA HMPREF0623_0 880	697	77041	ribosome disassembly [GO:0032790]	GTP binding [GO:0005525]; GTPase activity [GO:0003924]; translation elongation factor activity [GO:0003746]	cytosol [GO:0005829]; GTP binding [GO:0005525]; GTPase activity [GO:0003924]; translation elongation factor activity [GO:0003746]; ribosome disassembly [GO:0032790]	GO:0003746; GO:0003924; GO:0005525; GO:0005829; GO:0003746; GO:0032790
A0AAN5Y6X 6	Elongation factor G (EF-G)	fusA R0G89_07765	697	77013				
A0AAW8YM 53	Molecular chaperone DnaK	dnaK R0H03_03070	620	66923				
A0AAN5Y5 G9	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	dnaK R0G89_01095	618	66752				
E0NDH0	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	dnaK HMPREF0623_0 342	618	66738		ATP binding [GO:0005524]; ATP-dependent protein folding chaperone [GO:0140662]; unfolded protein binding [GO:0051082]	ATP binding [GO:0005524]; ATP-dependent protein folding chaperone [GO:0140662]; unfolded protein binding [GO:0051082]	GO:0005524; GO:0051082; GO:0140662

A0AAW8YP J0	Chaperonin GroEL	groL R0H03_07995	540	57557				
A0AAP3U15 2	Chaperonin GroEL (EC 5.6.1.7) (60 kDa chaperonin) (Chaperonin-60) (Cpn60)	groL groEL R0G89_05215	540	57499				
E0NI69	Chaperonin GroEL (EC 5.6.1.7) (60 kDa chaperonin) (Chaperonin-60) (Cpn60)	groL groEL HMPREF0623_1 700	540	57499	chaperone cofactor- dependent protein refolding [GO:0051085] ; protein refolding [GO:0042026] ; response to heat [GO:0009408]	ATP binding [GO:0005524]; ATP-dependent protein folding chaperone [GO:0140662]; isomerase activity [GO:0016853]; unfolded protein binding [GO:0051082]	GroEL-GroES complex [GO:1990220]; ATP binding [GO:0005524]; ATP-dependent protein folding chaperone [GO:0140662]; isomerase activity [GO:0016853]; unfolded protein binding [GO:0051082]; chaperone cofactor- dependent protein refolding [GO:0051085]; protein refolding [GO:0042026]; response to heat [GO:0009408]	GO:00055 24; GO:00094 08; GO:00168 53; GO:00420 26; GO:00510 82; GO:00510 85; GO:01406 62; GO:19902 20
E0NEA3	LysM domain protein	HMPREF0623_0 613	473	46919				
A0AAW8YE 57	LysM peptidoglycan- binding domain- containing protein	R0G89_02525	472	46820				
A0AAW8YF 52	30S ribosomal protein S1	rpsA R0G89_01980	403	44087				
A0AAW8YM C1	30S ribosomal protein S1	rpsA R0H03_02160	403	44059				
E0NDY6	30S ribosomal protein S1	rpsA HMPREF0623_0 508	403	44059	translation [GO:0006412]	mRNA binding [GO:0003729]; structural constituent of ribosome [GO:0003735]	cytosolic small ribosomal subunit [GO:0022627]; mRNA binding [GO:0003729]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]	GO:00037 29; GO:00037 35; GO:00064 12; GO:00226 27
A0AAW8YM R9	LysM peptidoglycan- binding domain- containing protein	R0H03_02685	432	43339				
A0AAW8YQ N8	Iron-containing alcohol dehydrogenase (EC 1.1.1.-)	R0H03_08655	389	42210				
A0AAW8YI2 2	Iron-containing alcohol dehydrogenase (EC 1.1.1.-)	R0G89_09180	389	42167				
E0NEK1	Alcohol dehydrogenase, iron-dependent (EC 1.1.1.1)	dhaT HMPREF0623_0 063	389	42164		alcohol dehydrogenase (NAD+) activity [GO:0004022];	alcohol dehydrogenase (NAD+) activity [GO:0004022];	GO:00040 22; GO:00468 72

					metal ion binding [GO:0046872]	metal ion binding [GO:0046872]		
A0AAP3TYI 4	Peptide chain release factor 1 (RF- 1)	prfA R0G89_07040 R0H03_07590	359	40995				
E0NF43	Peptide chain release factor 1 (RF- 1)	prfA HMPREF0623_0 773	359	40995	translation release factor activity, codon specific [GO:0016149]	cytoplasm [GO:0005737]; translation release factor activity, codon specific [GO:0016149]	GO:00057 37; GO:00161 49	
E0NGX4	Ribosome-binding ATPase YchF	ychF HMPREF0623_1 171	368	40439	ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]; GTP binding [GO:0005525]; ribosomal large subunit binding [GO:0043023]	cytoplasm [GO:0005737]; ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]; GTP binding [GO:0005525]; ribosomal large subunit binding [GO:0043023]	GO:00055 24; GO:00055 25; GO:00057 37; GO:00168 87; GO:00430 23	
A0AAN5YA2 0	Ribosome-binding ATPase YchF	ychF R0G89_03750	368	40408				
A0AAW8YJ Y7	Redox-regulated ATPase YchF	ychF R0H03_00865	368	40394				
A0AAW8YP R4	Type I glyceraldehyde-3- phosphate dehydrogenase	gap R0H03_08180	340	36643				
E0NI32	Glyceraldehyde-3- phosphate dehydrogenase (EC 1.2.1.12) (NAD- dependent glyceraldehyde-3- phosphate dehydrogenase)	gap HMPREF0623_1 663	340	36643	glucose metabolic process [GO:0006006]	glyceraldehyde-3- phosphate dehydrogenase (NAD+) (phosphorylating) activity [GO:0004365]; NAD binding [GO:0051287]; NADP binding [GO:0050661]	cytoplasm [GO:0005737]; glyceraldehyde-3- phosphate dehydrogenase (NAD+) (phosphorylating) activity [GO:0004365]; NAD binding [GO:0051287]; NADP binding [GO:0050661]; glucose metabolic process [GO:0006006]	GO:00043 65; GO:00057 37; GO:00060 06; GO:00506 61; GO:00512 87
A0AAN5YC 13	Glyceraldehyde-3- phosphate dehydrogenase (EC 1.2.1.12) (NAD- dependent glyceraldehyde-3- phosphate dehydrogenase)	gap R0G89_05030	340	36629				
A0AAP3UOF 2	Glycine--tRNA ligase alpha subunit (EC 6.1.1.14) (Glycyl- tRNA synthetase alpha subunit) (GlyRS)	glyQ R0G89_02090 R0H03_02270	299	34340				
E0NE08	Glycine--tRNA ligase alpha subunit (EC 6.1.1.14) (Glycyl- tRNA synthetase	glyQ HMPREF0623_0 530	299	34340	glycyl-tRNA aminoacylation [GO:0006426]	ATP binding [GO:0005524]; glycine-tRNA ligase activity	cytosol [GO:0005829]; ATP binding [GO:0005524];	GO:00048 20; GO:00055 24;

	alpha subunit) (GlyRS)					[GO:0004820]; transferase activity [GO:0016740]	glycine-tRNA ligase activity [GO:0004820]; transferase activity [GO:0016740]; glycyl-tRNA aminoacylation [GO:0006426]	GO:00058 29; GO:00064 26; GO:00167 40
A0AAP3U53 4	Elongation factor Ts (EF-Ts)	tsf R0G89_01005 R0H03_03160	292	31934				
E0NDF1	Elongation factor Ts (EF-Ts)	tsf HMPREF0623_0 323	292	31934		translation elongation factor activity [GO:0003746]	cytoplasm [GO:0005737]; translation elongation factor activity [GO:0003746]	GO:00037 46; GO:00057 37
A0AAN5Y8X 5	Fructose- bisphosphate aldolase	fba R0G89_07135	287	31070				
A0AAW8YP H3	Class II fructose-1,6- bisphosphate aldolase (EC 4.1.2.13)	fba R0H03_07685	287	31000				
E0NF61	Fructose- bisphosphate aldolase	fba HMPREF0623_0 791	287	31000	fructose 1,6- bisphosphate metabolic process [GO:0030388] ; glycolytic process [GO:0006096]	fructose- bisphosphate aldolase activity [GO:0004332]; zinc ion binding [GO:0008270]	fructose- bisphosphate aldolase activity [GO:0004332]; zinc ion binding [GO:0008270]; fructose 1,6- bisphosphate metabolic process [GO:0030388]; glycolytic process [GO:0006096]	GO:00043 32; GO:00060 96; GO:00082 70; GO:00303 88
A0AAW8YJ 70	50S ribosomal protein L2	rplB R0G89_07740	282	30722				
A0AAN5Y6J 5	Large ribosomal subunit protein uL2	rplB R0H03_05480	282	30694				
E0NFE5	Large ribosomal subunit protein uL2	rplB HMPREF0623_0 875	282	30694	cytoplasmic translation [GO:0002181]	rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; transferase activity [GO:0016740]	cytosolic large ribosomal subunit [GO:0022625]; rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; transferase activity [GO:0016740]; cytoplasmic translation [GO:0002181]	GO:00021 81; GO:00037 35; GO:00167 40; GO:00198 43; GO:00226 25
A0AAW8YJ T6	AAA family ATPase	R0G89_10710	263	30280				
A0AAN5YB5 6	Small ribosomal subunit protein uS2	rpsB R0G89_01000 R0H03_03165	261	29570				
E0NDF0	Small ribosomal subunit protein uS2	rpsB HMPREF0623_0 322	261	29570	translation [GO:0006412]	structural constituent of ribosome [GO:0003735]	cytosolic small ribosomal subunit [GO:0022627]; structural constituent of	GO:00037 35; GO:00064 12;

							ribosome [GO:0003735]; translation [GO:0006412]	GO:00226 27
P49668	Small ribosomal subunit protein uS2 (30S ribosomal protein S2)	rpsB	261	29570	translation [GO:0006412]	structural constituent of ribosome [GO:0003735]	cytosolic small ribosomal subunit [GO:0022627]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]	GO:00037 35; GO:00064 12; GO:00226 27
A0AAP3U1 R0	Amino acid ABC transporter ATP- binding protein	R0G89_05635 R0H03_05970	246	27725				
E0NI98	ABC transporter, ATP-binding protein (EC 3.6.3.-)	HMPREF0623_1 729	246	27725		ABC-type amino acid transporter activity [GO:0015424]; ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]	ABC-type amino acid transporter activity [GO:0015424]; ATP binding [GO:0005524]; ATP hydrolysis activity [GO:0016887]	GO:00055 24; GO:00154 24; GO:00168 87
A0AAW8YE A0	Thiazole synthase (EC 2.8.1.10)	R0G89_01800	257	27546				
A0AAW8YG Y2	16S rRNA (Guanine(527)-N(7))- methyltransferase RsmG (EC 2.1.1.170)	rsmG R0G89_03765	243	27110				
E0NGX9	Ribosomal RNA small subunit methyltransferase G (EC 2.1.1.-) (16S rRNA 7- methylguanosine methyltransferase) (16S rRNA m7G methyltransferase)	gidB rsmG HMPREF0623_1 176	243	27093		rRNA (guanine- N7-)- methyltransferase activity [GO:0070043]	cytosol [GO:0005829]; rRNA (guanine- N7-)- methyltransferase activity [GO:0070043]	GO:00058 29; GO:00700 43
A0AAW8YL H7	16S rRNA (Guanine(527)-N(7))- methyltransferase RsmG (EC 2.1.1.170)	rsmG R0H03_00880	243	27063				
A0AAW8YH T6	(S)-acetoin forming diacetyl reductase (EC 1.1.1.304)	R0G89_06315	258	26981				
E0NHS7	Diacetyl reductase [(S)-acetoin forming] (EC 1.1.1.304) (Acetoin(diacetyl) reductase) (Meso- 2,3-butanediol dehydrogenase)	budC HMPREF0623_1 843	258	26938	acetoin catabolic process [GO:0045150]	diacetyl reductase ((S)-acetoin forming) (NAD+) activity [GO:0052588]	diacetyl reductase ((S)-acetoin forming) (NAD+) activity [GO:0052588]; acetoin catabolic	GO:00451 50; GO:00525 88

					process [GO:0045150]			
A0AAW8YN U6	(S)-acetoin forming diacetyl reductase (EC 1.1.1.304)	R0H03_06500	258	26919				
A0AAP3U1 H0	2,3- bisphosphoglycerate -dependent phosphoglycerate mutase (BPG- dependent PGAM) (PGAM) (Phosphoglyceromut ase) (dPGM) (EC 5.4.2.11)	gpmA R0G89_03660 R0H03_00795	229	26029				
E0NGW1	2,3- bisphosphoglycerate -dependent phosphoglycerate mutase (BPG- dependent PGAM) (PGAM) (Phosphoglyceromut ase) (dPGM) (EC 5.4.2.11)	gpm2 gpmA HMPREF0623_1 158	229	26029	gluconeogene sis [GO:0006094] ; glycolytic process [GO:0006096]	2,3- bisphosphoglycer ate-dependent phosphoglycerate mutase activity [GO:0046538]	2,3- bisphosphoglycer ate-dependent phosphoglycerate mutase activity [GO:0046538]; gluconeogenesis [GO:0006094]; glycolytic process [GO:0006096]	GO:00060 94; GO:00060 96; GO:00465 38
A0AAN6BH P8	2,3- bisphosphoglycerate -dependent phosphoglycerate mutase (BPG- dependent PGAM) (PGAM) (Phosphoglyceromut ase) (dPGM) (EC 5.4.2.11)	gpmA R0G89_08175	223	25466				
A0AAW8YL B8	2,3- bisphosphoglycerate -dependent phosphoglycerate mutase (EC 5.4.2.11)	R0H03_05040	223	25368				
A0AAP3X7B 2	Cytidylate kinase (CK) (EC 2.7.4.25) (Cytidine monophosphate kinase) (CMP kinase)	cmk R0G89_01985	226	25210				
A0AAW8YK M7	(D)CMP kinase (EC 2.7.4.25)	cmk R0H03_02165	226	25196				
E0NDY7	Cytidylate kinase (CK) (EC 2.7.4.25) (Cytidine monophosphate kinase) (CMP kinase)	cmk HMPREF0623_0 509	226	25196	nucleobase- containing small molecule interconversio n [GO:0015949] ; phosphorylati on [GO:0016310] ; pyrimidine nucleotide metabolic process [GO:0006220]	ATP binding [GO:0005524]; CMP kinase activity [GO:0036430]; dCMP kinase activity [GO:0036431]	cytosol [GO:0005829]; ATP binding [GO:0005524]; CMP kinase activity [GO:0036430]; dCMP kinase activity [GO:0036431]; nucleobase- containing small molecule interconversion [GO:0015949]; phosphorylation [GO:0016310]; pyrimidine	GO:00055 24; GO:00058 29; GO:00062 20; GO:00159 49; GO:00163 10; GO:00364 30; GO:00364 31

							nucleotide metabolic process [GO:0006220]	
A0AAW8YJ G3	Ribose-5-phosphate isomerase RpiA (EC 5.3.1.6)	rpiA R0G89_08165	228	25016				
A0AAW8YM 83	Ribose-5-phosphate isomerase RpiA (EC 5.3.1.6)	rpiA R0H03_05050	228	24985				
E0NFM5	Ribose-5-phosphate isomerase A (EC 5.3.1.6) (Phosphoriboisomer ase A) (PRI)	rpiA HMPREF0623_0 955	228	24985	D-ribose metabolic process [GO:0006014] ; pentose- phosphate shunt, non- oxidative branch [GO:0009052]	ribose-5- phosphate isomerase activity [GO:0004751]	cytosol [GO:0005829]; ribose-5- phosphate isomerase activity [GO:0004751]; D- ribose metabolic process [GO:0006014]; pentose- phosphate shunt, non-oxidative branch [GO:0009052]	GO:00047 51; GO:00058 29; GO:00060 14; GO:00090 52
A0AAW8YI5 5	LysM peptidoglycan- binding domain- containing protein	R0G89_07155	226	23921				
A0AAN6BI8 4	Guanylate kinase (EC 2.7.4.8) (GMP kinase)	gmk R0G89_00555 R0H03_03430	204	23427				
E0NEZ9	Guanylate kinase (EC 2.7.4.8) (GMP kinase)	gmk HMPREF0623_0 211	204	23427	phosphorylati on [GO:0016310]	ATP binding [GO:0005524]; guanylate kinase activity [GO:0004385]	cytosol [GO:0005829]; ATP binding [GO:0005524]; guanylate kinase activity [GO:0004385]; phosphorylation [GO:0016310]	GO:00043 85; GO:00055 24; GO:00058 29; GO:00163 10
A0AAN6BH G7	Large ribosomal subunit protein uL4	rplD R0H03_05470	207	22751				
E0NFE7	Large ribosomal subunit protein uL4	rplD HMPREF0623_0 877	207	22751	translation [GO:0006412]	rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]	ribonucleoprotein complex [GO:1990904]; ribosome [GO:0005840]; rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]	GO:00037 35; GO:00058 40; GO:00064 12; GO:00198 43; GO:19909 04
A0AAW8YJ 88	50S ribosomal protein L4	rplD R0G89_07750	207	22737				
A0AAP3TYP 9	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92) (Endopeptidase Clp)	clpP R0G89_05050 R0H03_08160	197	21509				
E0NI36	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92) (Endopeptidase Clp)	clpP HMPREF0623_1 667	197	21509	protein quality control for misfolded or incompletely synthesized	ATP-dependent peptidase activity [GO:0004176]; ATPase binding [GO:0051117]; serine-type	cytoplasm [GO:0005737]; endopeptidase Clp complex [GO:0009368]; ATP-dependent	GO:00041 76; GO:00042 52; GO:00057 37;

					proteins [GO:0006515]	endopeptidase activity [GO:0004252]	peptidase activity [GO:0004176]; ATPase binding [GO:0051117]; serine-type endopeptidase activity [GO:0004252]; protein quality control for misfolded or incompletely synthesized proteins [GO:0006515]	GO:00065 15; GO:00093 68; GO:00511 17
E0NDF3	Ribosome-recycling factor (RRF) (Ribosome-releasing factor)	frr HMPREF0623_0 325	186	20690	translational termination [GO:0006415]	ribosomal large subunit binding [GO:0043023]	cytoplasm [GO:0005737]; ribosomal large subunit binding [GO:0043023]; translational termination [GO:0006415]	GO:00057 37; GO:00064 15; GO:00430 23
A0AAP3XA A7	Ribosome-recycling factor (RRF) (Ribosome-releasing factor)	frr R0G89_01015 R0H03_03150	186	20662				
A0AAN5Y6 W9	Large ribosomal subunit protein uL5	rplE R0G89_07695 R0H03_05525	180	20179				
E0NFD6	Large ribosomal subunit protein uL5	rplE HMPREF0623_0 866	180	20179	translation [GO:0006412]	rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; tRNA binding [GO:0000049]	cytosolic large ribosomal subunit [GO:0022625]; rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; tRNA binding [GO:0000049]; translation [GO:0006412]	GO:00000 49; GO:00037 35; GO:00064 12; GO:00198 43; GO:00226 25
P37249	Pediocin PA-1 biosynthesis protein PedC (Pediocin ACH biosynthesis protein PapC)	pedC papC	174	19194	bacteriocin biosynthetic process [GO:0030152]		bacteriocin biosynthetic process [GO:0030152]	GO:00301 52
Q68GC4	PedC		174	19194	bacteriocin biosynthetic process [GO:0030152]		bacteriocin biosynthetic process [GO:0030152]	GO:00301 52
A0AAN5Y9B 2	Small ribosomal subunit protein uS7	rpsG R0G89_07770 R0H03_05450	156	17948				
E0NFF1	Small ribosomal subunit protein uS7	rpsG HMPREF0623_0 881	156	17948	ribosomal small subunit assembly [GO:0000028] ; translation [GO:0006412]	mRNA binding [GO:0003729]; rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; tRNA binding [GO:0000049]	cytosolic small ribosomal subunit [GO:0022627]; mRNA binding [GO:0003729]; rRNA binding [GO:0019843]; structural constituent of ribosome	GO:00000 28; GO:00000 49; GO:00037 29; GO:00037 35; GO:00064 12;

							[GO:0003735]; tRNA binding [GO:0000049]; ribosomal small subunit assembly [GO:0000028]; translation [GO:0006412]	GO:00198 43; GO:00226 27
A0AAP3TZ0 3	Transcription elongation factor GreA (Transcript cleavage factor GreA)	greA R0G89_00375 R0H03_03610	161	17872				
E0NEW1	Transcription elongation factor GreA (Transcript cleavage factor GreA)	greA HMPREF0623_0 173	161	17872	DNA- templated transcription elongation [GO:0006354] ; regulation of DNA- templated transcription elongation [GO:0032784]	DNA binding [GO:0003677]; RNA polymerase binding [GO:0070063]; translation elongation factor activity [GO:0003746]	DNA binding [GO:0003677]; RNA polymerase binding [GO:0070063]; translation elongation factor activity [GO:0003746]; DNA-templated transcription elongation [GO:0006354]; regulation of DNA-templated transcription elongation [GO:0032784]	GO:00036 77; GO:00037 46; GO:00063 54; GO:00327 84; GO:00700 63
A0AAN6BF U9	Small ribosomal subunit protein uS5	rpsE R0G89_07670 R0H03_05550	167	17579				
E0NFD1	Small ribosomal subunit protein uS5	rpsE HMPREF0623_0 861	167	17579	translation [GO:0006412]	rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]	cytoplasm [GO:0005737]; small ribosomal subunit [GO:0015935]; rRNA binding [GO:0019843]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]	GO:00037 35; GO:00057 37; GO:00064 12; GO:00159 35; GO:00198 43

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Manuscrito VI

Extending the shelf life of plant-based meat analogues by adding cell-free supernatant from *Lactiplantibacillus plantarum*

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Article

Extending the shelf life of plant-based meat analogues by adding cell-free supernatant from *Lactiplantibacillus plantarum*

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Abstract:

The growing demand for sustainable and clean-label food products has driven the development of plant-based meat analogues with extended shelf life and safety. This study evaluates the application of the cell-free supernatant (CFS) derived from *Lactiplantibacillus plantarum* CNTA 600 as a natural biopreservative in soy-based meat analogues. The strain was characterized through whole genome sequencing, confirming its taxonomic identity, absence of acquired antibiotic resistance, and presence of genes encoding plantaricin bacteriocins. Protein profiling including SDS-PAGE, zymography, and mass spectrometry, validated the production of antimicrobial peptides with activity against plant-based meat analogues spoilage microorganisms identified as *Niallia circulans* and *Bacillus cereus* group. The CFS was incorporated into the formulation of plant-based bites, replacing water in the preconditioning process which were then subjected to marinating, thermal treatment and vacuum packaging. Microbiological and physicochemical analyses revealed that the CFS-treated samples maintained microbial stability and sensory quality throughout storage (more than 90 days), while control samples exceeded microbial limits after 65 days. The addition of the CFS did not significantly alter pH or sensory attributes, supporting its suitability for clean-label applications. These findings supported the use of the CFS of *L. plantarum* CNTA 600 as a promising natural preservative capable of extending the shelf life of plant-based meat analogues from 30 to 95 days, offering a sustainable alternative to synthetic additives.

Keywords: plant-based meat analogues, *Lactiplantibacillus plantarum*, biopreservation, shelf-life extension, plantaricin, cell-free supernatant (CFS).

1. INTRODUCTION

The consumption of meat, particularly red and processed meat, has been linked to increased health risks and significant environmental impacts (Corentin Biteau, 2025). Plant-based products are emerging as a sustainable alternative to meat and are projected to grow significantly, with a compound annual growth rate (CAGR) of 12% in Europe (2021-2028), 18.5% in Asia-Pacific region (2021-2027), and over 17.5% in the United States during the same period (Talwar et al., 2024). They are gaining momentum as sustainable alternatives, becoming more competitive with traditional animal products like meat, egg, fish, and dairy (Boukid, 2021; Pointke & Pawelzik, 2022). Analogues, defined as compounds structurally like others but differing in composition, aim to mimic the taste, texture, and nutritional profile with fewer environmental impacts (Ismail et al., 2020; Joshi & Satish Kumar, 2015; Singh & Sit, 2022; Suna et al., 2021).

The supply of analogues includes diverse options, with meat and dairy analogues being the most prominent (Bohrer, 2019; Ismail et al., 2020; Suna et al., 2021). Plant-based meat alternatives, made from soy, mushrooms, wheat protein, or legumes, are the most popular due to lower production costs and higher consumer acceptance (Astiasarán et al., 2023; Kołodziejczak et al., 2021; Suna et al., 2021). However, not all plant-based ingredients can faithfully mimic the fiber, flavour and taste of meat (Florian Wild, 2016). Textured soy protein performs best in terms of these properties. These analogues are produced using low-moisture and high-moisture extrusion techniques, with high-moisture extrusion creating products that closely mimic traditional meat (Chakravorty & Das, 2024; Florian Wild, 2016).

However, these soy-based analogues are susceptible to contamination by bacteria, moulds or yeasts through untreated raw ingredients or through environmental contamination (Liu et al., 2023). These microorganisms can survive or even grow in meat

analogues, shortening their shelf life or even posing a food safety risk, which makes the addition of preservatives necessary. However, consumers increasingly demand clean label products, which is driving the search for natural alternatives to the approved list of additives (Bohrer, 2019; Ismail et al., 2020; Mishal et al., 2022).

To meet clean label requirements, companies are adopting alternatives to conventional preservation methods to extend product shelf life. One prominent trend is the use of protective cultures as natural and sustainable preservatives, known as biopreservation. These cultures can extend the shelf life of products by producing antimicrobial compounds such as bacteriocins and organic acids (Udayakumar et al., 2022; Yang et al., 2024). Various methods exist for incorporating these microorganisms or their antimicrobial compounds into products, including cell-free supernatants (CFS) (Garin-Murguialday et al., 2024). *Lactiplantibacillus plantarum* is one of the most cited and significant species as protective culture, with applications ranging from fresh-cut fruits and vegetables (de Simone et al., 2024; Wong et al., 2021) to animal-based products like cheese (Engstrom et al., 2021; Khalil et al., 2022), meat (Arrioja-Bretón et al., 2020; Barcenilla et al., 2023; Jo et al., 2021) or seafood (Jo et al., 2021).

(Garin-Murguialday et al., 2024) identified and characterized the strain *Lactiplantibacillus plantarum* CNTA 600 as a promising protective culture. This strain meets the stringent requirements set by the European Commission for food industry microorganisms. Its CFS demonstrated significant antimicrobial activity against the gram-positive bacteria *Listeria monocytogenes* CECT 7467, attributed to the production of bacteriocins. Furthermore, the heat resistance of its CFS allowed its application in pasteurized products, thereby expanding its potential use in various food applications. (Sun et al., 2021)

In this context, the antimicrobial potential of the CFS of *Lactiplantibacillus plantarum* offers a promising solution to extend the shelf life of plant-based analogues, providing a natural and clean label approach to food preservation. Moreover, shelf-life extension has a direct positive impact on business economics. For companies, increasing shelf life from 30 to 90 days can significantly reduce Food waste, improve supply chain efficiency, and open access to broader markets, all without compromising the product's green image (Casson et al., 2022).

The aim of this study is to characterize the *L. plantarum* CNTA 600 strain using bioinformatic, molecular, and *in vitro* analysis, and to validate its application in a real plant-based product by demonstrating both its sensory acceptability and its effectiveness in extending shelf life from 30 to 90 days.

2. MATERIALS AND METHODS

2.1. Protective Culture Characterization

2.1.1. Whole Genome Sequencing of *L. plantarum* CNTA 600

Illumina iSeq100 platform (KIMITEC, Almeria, Spain) was used to sequence the genome of the strain *Lactiplantibacillus plantarum* CNTA 600. DNA extraction was performed using the Zymo Quick-DNA Fungal/Bacterial Miniprep kit and the Quant-iT dsDNA Assay Kit (Thermo Fisher, MA, USA) was used to quantify it. The genomic DNA extracted was subsequently utilized to create a sequencing library using the Illumina DNAprep kit (Illumina). The fragment distribution within the library was examined with the Bioanalyzer 2100 (Agilent Technologies, California, USA).

Raw 2×150 bp paired-end reads were quality-checked using FastQC v.0.12.1 (Andrews, 2010) and adapters and low-quality reads were filtered with Trim Galore v.0.6.10. De-novo genome assemblies were generated using multiple assemblers,

including SPAdes v.3.15.5 (Bankevich et al., 2012), shovill v0.9.0 (Seemann, 2017), and a5-miseq GPLv3 (Coil et al., 2015). Based on comparative assessment of assembly quality with QUAST v5.2.0 (e.g., contiguity and completeness), the shovill-based assembly was selected for downstream analyses (Gurevich et al., 2013).

The previous taxonomic assignment performed via Sanger sequencing of the 16S rRNA gene was confirmed by analyzing clean reads using Kraken2 v.2.1.3 (Wood et al., 2019), and by comparison of the assembled genome to a closely related reference genome (NCBI RefSeq assembly GCF_000143745.1) via calculation of the Average Nucleotide Identity using FastANI v1.33 (Jain et al., 2018).

Antimicrobial resistance genes were detected using the Resistance Gene Identifier (RGI) tool v6.0.3 with the CARD v4.0.0 database (Alcock et al., 2023), BacAnt (Hua et al., 2021), and ABRicate v1.0.1 (Seeman, 2016) with comprehensive screening across the CARD, MEGARes 2.0, NCBI Bacterial Antimicrobial Resistance Reference Gene Database, ResFinder v4, and ARG-ANNOT databases. To maximize sensitivity, an initial relaxed threshold of 5% identity and coverage was applied to capture all potential hits, followed by manual filtering to retain only high confidence matches with $\geq 80\%$ identity and $\geq 70\%$ coverage ((EFSA), 2024b). PathogenFinder v1.1 (Cosentino et al., 2013) was used to predict the pathogenic potential of the isolate based on genomic features.

To predict the type and location of secondary metabolite biosynthesis gene clusters within the assembled genomes, genome annotation was performed with Prokka v.1.14.6 (Seemann, 2014), and the resulting annotated sequences were queried against the BAGEL4 database (van Heel et al., 2018) and the APD3 (Antimicrobial Peptide Database) (Wang et al., 2016) using BLAST+ (Camacho et al., 2009) with a minimum identity threshold of 80%.

2.1.2. Protein-based characterization of the cell-free supernatant

2.1.2.1. Cell-Free Supernatant Preparation

Based on the study by (Garin-Murguialday et al., 2024), protective cultures for plant-based bites were selected from the bacterial collection of the National centre for Food Technology and Safety (CNTA) in Navarra, Spain. Four strains were initially considered for their antimicrobial properties. Ultimately, *Lactiplantibacillus plantarum* CNTA 600 were chosen as potential protective culture for the meat analogue. The antimicrobial activity of this strain against spoilage organisms in plant-based bites was evaluated using its cell-free supernatants (CFS). The CFS of each strain was produced following the methodology outlined by (Garin-Murguialday et al., 2024) in liquid format.

2.1.2.2. Molecular characterization by SDS-PAGE and Zymography of the CFS

The SDS-PAGE technique was used to determine the protein profile of the strain and the molecular weight of the proteinaceous antimicrobial compounds. To purify the bacteriocins, a 1 L suspension of the protective culture was incubated for 24 hours, centrifuged, and filtered to obtain its CFS (Garin-Murguialday et al., 2024). It was treated with 80% ammonium sulphate (Merck) and incubated overnight at 10°C. The mixture was then centrifuged at $10,000 \times g$ for 10 min in an Eppendorf centrifuge 5910 Ri (Eppendorf, Hamburg, Germany), the supernatant discarded, and the biomass resuspended in phosphate buffered saline (PBS) (OXOID, Madrid, Spain). Membrane dialysis was performed with two molecular weight cut-off (MWCO) membranes (Thermo Fisher), and the dialyzed proteins were collected.

A 16% acrylamide gel was prepared, and electrophoresis was conducted following a modified SDS-PAGE Tris/Tricine protocol described by (Schägger, 2006). The dialyzed proteins were treated with dithiothreitol (DTT) (PanReac, Barcelona, Spain) and sodium dodecyl sulphate (SDS) (PanReac), heated to induce denaturation, and loaded onto the

gel along with molecular weight markers (PageRuler™ Prestained Protein Ladder, 10-180 kDa, Ref.: 26616 (Thermo Fisher), and Spectra™ Multicolour Low Range Protein Ladder, Ref.: 26628 (Thermo Fisher)). Electrophoresis was conducted at 60 V for 5 hours with the Mini-PROTEAN Tetra Electrophoresis Cell (BIO RAD, Berkley, MI, USA). The gel was then fixed for 30 minutes with a mix of 50% ethanol, 10% glacial acetic acid, and 40% sterile distilled water, stained with Coomassie solution (Dyballa & Metzger, 2009), and destained the next day. The bands were compared with the markers and analyzed using ImageJ (BIO RAD, Berkley, MI, USA).

To identify proteins with antimicrobial activity and determine their molecular weight, the zymography technique was used in parallel. The sample of *Lactiplantibacillus plantarum* CNTA 600 was placed on different petri dishes (VWR, Radnor, PA, USA) and overlaid with indicator microorganisms *Listeria monocytogenes* CECT 7467, *Bacillus cereus* CECT 193 and *Bacillus cereus* group CNTA 719 (isolated from quinoa) at a concentration of 10^6 CFU/m. The plate was refrigerated at 8°C for 6 hours, followed by aerobic incubation at 37°C. After 17 hours, the inhibition halos were compared with the bands on the stained gel using the SDS-PAGE method.

2.1.2.3. Mass spectrometry

The liquid chromatography-mass spectrometry (LC-MS) analysis was performed with an Evosep One system (Evosep Biosystems, Odense, Denmark) connected to a ThermoFisher Scientific Exploris Orbitrap 480 mass spectrometer. The previously lyophilized peptides were used to carry out the analysis, which were reconstituted with 0.1% formic acid and then quantified using a NanoDrop spectrophotometer (ThermoFisher). 50g of these peptides were loaded in an Evotip and their resolution was conducted using a C18 Aurora Ultimate column (75 µm x 15 cm, particle size of 1.7 µm) (IonOpticks, Melbourne, Australia) employing the WhisperZoom method for 20 samples

per day (Evosep Biosystems). Data were collected in data-dependent acquisition (DDA) mode, applying defined parameters for both MS and MS/MS scans and peptide fragmentation was achieved using higher-energy collisional dissociation (HCD) with a normalized collision energy of 30%.

The acquired data were processed by Spectromine software (v 4.5.240625.52329) (Biognosys, Zurich, Switzerland), incorporating dynamic calibration for both MS1/MS2 levels, along with specified modifications. Trypsin was set as the proteolytic enzyme, and a false discovery rate (FDR) threshold of 1% was applied. The dataset was searched against a FASTA-formatted file containing *Lactiplantibacillus plantarum* CNTA 600 protein sequences.

2.2. Identification of spoilage microorganisms and *in-vitro* evaluation of the antimicrobial activity of the CFS

To develop an appropriate preservation strategy for plant-based bites, the product was analyzed along with the factors contributing to the termination of its shelf-life.

2.2.1. Detection and Analysis of Spoilage Microorganisms in Plant-Based Bites

The product used to test *in situ* the antimicrobial activity of the CFS of *L. plantarum* CNTA 600 consisted of plant-based bites, formulated from soy protein and marinated with a blend of selected species. To identify the cause of its shelf-life expiration, three trays of the final product were subjected to accelerated spoilage by storing them at 15°C for 15 days according to what described by (Amit et al., 2017) in their work on the mechanisms and commercial aspects of preservation in the food industry; they described the type of preservation that should be carried out, as well as the unfavourable conditions for different types of food.

When this time ended, each tray was independently homogenised seeded, with each being a separate replicate. After the incubation period, ten grams of the plant-based

bites were taken from each tray and homogenized using a stomacher homogenizer blender AES Easy Mix (QuestPair, The Netherlands) with 0.1% buffered peptone water (BPW) (Merck). After homogenizing and filtering the product through VWR® Blender stomacher bags with lateral filter (VWR, Radnor, PA, USA), various dilutions were prepared using 0.1% BPW. These dilutions were then plated onto different media (Yousuf & Srivastava, 2017) TSA-YE (recovery of aerobic mesophiles), MRS agar (recovery of lactic acid bacteria) and DRBC agar (recovery of moulds and yeasts) and incubated under specific conditions tailored to each medium.

Following colony isolation, taxonomic identification was conducted for each isolate using the methodology described by (Garin-Murguialday et al., 2024), in which bacterial identification was performed through 16S rRNA gene sequencing, and fungal identification through sequencing of the D1/D2 region of the 26S rDNA.

2.2.2. *In vitro* antimicrobial activity of the CFS

Following the identification of the microorganisms responsible for the spoilage of chicken-type snacks, the *in vitro* antimicrobial activity of the CFS of *Lactiplantibacillus plantarum* strain CNTA 600 was assessed. This was achieved using the agar well diffusion method and time-kill curve analysis, through which the minimum inhibitory concentration (MIC) was determined. Both methodologies employed in this study follow the protocols described by (Garin-Murguialday et al., 2024), with some changes.

2.2.2.1. Antimicrobial activity of the CFS by Agar Well Diffusion Assay

In this procedure, 100 µL of the CFS was used and faced against a final plate concentration of 10⁶ CFU/mL of each spoilage microorganism. The plates were then kept at room temperature in the laboratory for 2 hours before being incubated under the optimal growth conditions for the wild-type microorganisms.

2.2.2.2. Antimicrobial activity of the CFS by Time-Kill Curve Assay and the Minimum Inhibitory Concentration (MIC)

To gain a deeper understanding of the antimicrobial properties displayed by the CFS of the chosen strain, and to describe the dynamic interaction and antimicrobial activity over time against the spoilage microorganisms, a time-kill curve assay was performed (Garin-Murguialday et al., 2024) to determine the MIC (% v/w) of the selected CFS that, when added to the ingredient mixture of the bites prior to extrusion, would ensure antibacterial activity against the spoilage microorganisms responsible for the end of the shelf life. This involved using a sterile 96-well flat-bottom microtiter plate with increasing concentrations of the CFS (0%, 0.31%, 0.63%, 1.25%, 2.5% y 5% v/v) against a final well concentration of 10^3 CFU/mL of the indicator microorganisms. The plates were incubated under optimal growth conditions (during 28 hours at 37°C) and monitored visually and using a plate reader every 2 hours.

2.3. *In Situ* Assessment of CFS Application in Plant-Based Chicken Bites

To conduct the trial, three distinct batches of plant-based bites were prepared: a control batch following the company's standard procedure, a batch incorporating the cell-free supernatant (CFS) of *Lactiplantibacillus plantarum*. The manufacturing process for all batches commenced with wet extrusion, preceded by the preparation of soy protein, which is the step where the products differ.

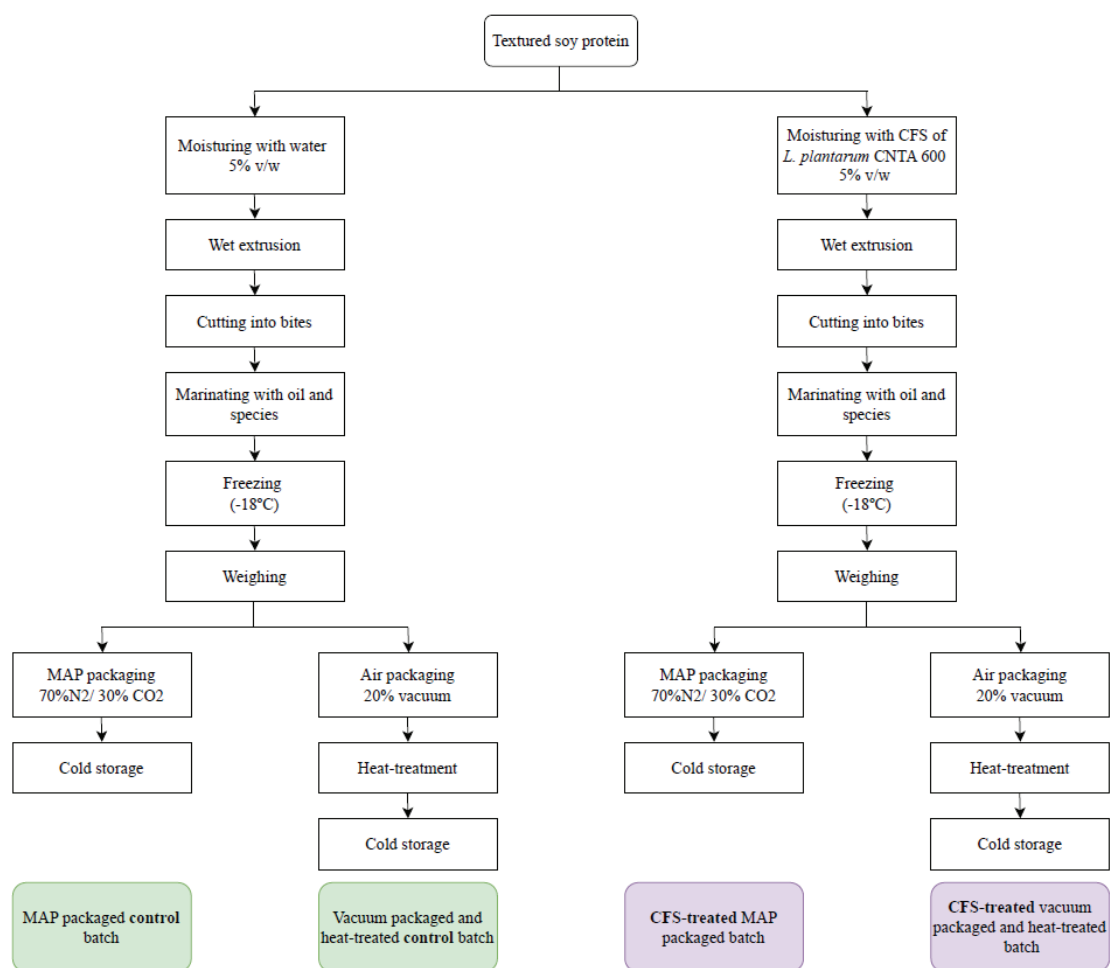


Figure 1. Designed and followed flowchart for the production of the different batches of plant-based bites

2.3.1. Production of plant-based chicken bites

Figure 1 illustrates the process of preparing the control batch of test bites. Initially, soy protein was moistened with water (5% v/w) as a preconditioning step to facilitate the wet extrusion. The extrusion was conducted by an equipment formed by the extruder B-TSE-S 20/40 (TwinLab, Florida, United States) model, a hopper model DDW-M-DDSR20 (Kubota Brabender Technologie, Duisburg, Germany). Post-extrusion, the soy product was vacuum-packed and rapidly cooled using a blast chiller (ABT14 1L, INFIRCO, Córdoba, Spain) at 4°C. Subsequently, under refrigeration temperatures in aseptic conditions the extruded formula was cut into bites (2.5 x 5.8 cm) and marinated with mediterranean spices, oil, and salt. To replicate the company's full production process, the bites were frozen at -18°C and packed in plastic PP/EVOH/PP trays and

PP/EVOH/PP film under two different atmospheric conditions: a modified atmosphere (MAP) with 70% N₂ and 30% CO₂, and air with a 20% vacuum. The trays packed in MAP conditions were directly stored at 4°C for 30 days and at 8°C for 60 days, while the batch packed in 20% vacuum was heat treated at 85°C for 15 minutes before being stored at 4°C for 30 days and at 8°C for 60 days to simulate distribution conditions involving cold chain fluctuations at retail (Mui et al., 2025).

The CFS from *L. plantarum* strain CNTA 600 was incorporated into the soy protein preparation, replacing the water used in the previous batches. Following this initial step, the processing was identical to that described in section 2.3.1.1., resulting in two sub-batches of snacks packaged in different atmospheres and heat treated. These were then stored under varying refrigeration conditions, depending on the product's shelf life.

2.3.2. Sensory Assessment of Plant-Based Bites by Consumer Panel

A panel of 11 judges, composed of technicians and researchers from the CNTA, conducted the sensory evaluation exclusively at time 0 days, after the production of the plant-based bites. The primary objective of the test was to determine whether the addition of the CFS from a bacteria strain produced a significant effect of change in the plant-based bites, as well as to assess whether the panellists were able to perceive such changes, if were, in the final product.

With that purpose, the sensory analysis was conducted with a 2-day delay after production, as the sensory evaluation could not be performed until pathogen testing confirmed the absence of microbiological risk following the sampling period. The bites from the two batches were presented cooked, as this reflects their typical mode of consumption, and served on white plates to avoid influencing colour perception.

The evaluation form provided to the panellists was divided into the parameters of colour, odour, texture and flavour. Panellists were asked to indicate whether each attribute was “typical” or “not typical” in comparison to a reference product of the same characteristics (plant-based bites) purchased from a supermarket.

2.3.3. Physicochemical parameters of the plant-based bites

2.2.3.1. pH determination

The pH determination of each sample was done by taking an aliquot of the stomached bites and measuring them by the pHmeter GLP21 (CRISON, Barcelona, Spain).

2.2.3.2. Trays atmospheric composition

At each sampling point, the atmospheric composition of three replicate trays containing the bites was measured. This analysis included trays packed in air with a 20% vacuum and those packed in a modified atmosphere (MAP) with 80% CO₂ and 20% N₂. The measurements were conducted with an automatic gas analyser (PBI, Dansensor; Barcelona, Spain), utilizing a syringe through an adhesive septum on the film cover.

2.3.4. Microbial Characterization of Spoilage Populations in Plant-Based Bites

The method employed were consistent with those mentioned by (Garin-Murguialday et al., 2024). The bites were homogenized in BPW at 0.1% (Merck) with the AES Easy Mix (QuestPair, The Netherlands, Europe) and subjected to serial 1:10 dilutions. These dilutions were then surface-plated on TSA-YE plates (Scharlau), DRBC (Scharlau) and MRSA (Merck).

2.4. Statistical analysis

All figures were created using GraphPad PRISMA software (version 10.3.0). Statistical analyses assumed normal distribution within each group. Two-way ANOVA test was employed to assess differences across all time points and treatments for

physicochemical and microbial parameters. Statistical significance was defined as $p \leq 0.05$. When a significant main effect was detected, post hoc pairwise comparisons were performed using Tuckey test, with significance set at $p \leq 0.05$. Additionally, microbial growth levels between the control and the treated group were compared with independent-samples *t-tests* for specific timepoints ($p \leq 0.05$).

All figures were created using GraphPad PRISMA software (version 10.3.0). Statistical analyses assumed normal distribution within each group. Two-way ANOVA test was employed to assess differences across all time points for physicochemical and microbial parameters across all time points and tested treatments. Statistical significance was defined as $p \leq 0.05$. When a significant main effect was detected, post hoc pairwise comparisons were performed using Tuckey test, with significance set at $p \leq 0.05$. Additionally, microbial growth levels between the control and the treated group were compared with independent-samples *t-tests* for specific timepoints ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1. Molecular characterization of *L. plantarum* CNTA 600

3.1.1. Whole Genome Sequencing of the strain *L. plantarum* CNTA 600

Whole Genome Sequencing was performed to confirm the taxonomic identity of the isolate, assess the absence of acquired antibiotic resistance and pathogenicity determinants, and detect biosynthetic cluster genes related to the production of bacteriocins or other secondary metabolites. This genomic evaluation aligns with EFSA recommendations ((EFSA), 2024a) for the use of microbial strains in food applications. WGS-based taxonomic classification of the isolate was conducted using Kraken2, which identified it as *Lactiplantibacillus plantarum*, a species included in the EFSA Qualified Presumption of Safety (QPS) list (EFSA Panel on Biological Hazards (BIOHAZ), 2025). In addition, FastANI analysis revealed an average nucleotide identity (ANI) of 98.98%

with the reference genome of *L. plantarum* ATCC 14917, exceeding the species delineation threshold of 95%, therefore supporting its classification within the same species.

Regarding the search for antibiotic resistance genes, none of the low-confidence hits (Table S1) exceeded the high-confidence filtering threshold ($\geq 80\%$ identity, $\geq 70\%$ coverage). These results, together with the previously performed phenotypic MIC testing (Garin-Murguialday et al., 2024), showed that *L. plantarum* CNTA 600 did not appear to carry acquired antibiotic resistance determinants of concern. Furthermore, the strain was deemed not pathogenic to humans after evaluation with PathogenFinder (prediction score of -37.899).

Table 1. Antibiotic resistance genes detected in the assembled genome of *Lactiplantibacillus plantarum* CNTA 600 using multiple analytical tools under relaxed thresholds ($\geq 5\%$ sequence coverage and identity). The table includes the corresponding tool and database, genomic locations, and the respective coverage and identity percentages.

Tool	Database	Gene	%Coverage	%Identity
RGI	CARD	<i>vanY</i>	95.90	31.93
Abricate	CARD	<i>Bifidobacterium_adolescentis_r poB_conferring_resistance_to_r ifampicin</i>	10.31	69.27
Abricate	megares	<i>lmrD</i>	31.73	67.29
BacAnt	resDB	<i>bpsD</i>	52.83	66.76
BacAnt	resDB	<i>cadC</i>	53.28	71.8
BacAnt	resDB	<i>arsB_pI258</i>	87.13	68.5
BacAnt	resDB	<i>bpsX</i>	20.68	68.31
BacAnt	resDB	<i>bpsX</i>	27.36	71.19
BacAnt	resDB	<i>bpsX</i>	22.75	73.76
BacAnt	resDB	<i>vanR-F</i>	31.47	68.49
BacAnt	resDB	<i>bpsX</i>	9.47	5.58
BacAnt	resDB	<i>cesA</i>	1.84	70.43
BacAnt	resDB	<i>bpsD</i>	9.97	80.6
BacAnt	resDB	<i>tetA(58)</i>	24.42	68.65
BacAnt	resDB	<i>taeA</i>	11.66	67.4
BacAnt	resDB	<i>vanC2/3</i>	8.93	75.53
BacAnt	resDB	<i>cesD</i>	8.11	78.87
BacAnt	resDB	<i>tetB(60)</i>	6.95	77.69

BacAnt	resDB	<i>tetA(60)</i>	7.36	70.31
BacAnt	resDB	<i>tetB(46)</i>	10.94	68.42
BacAnt	resDB	<i>copA_Eh</i>	5.17	73.45
BacAnt	resDB	<i>copA_Eh</i>	8.88	68.04
BacAnt	resDB	<i>copB_Eh</i>	5.9	70.45
BacAnt	resDB	<i>copB_Eh</i>	20.87	64.88

Functional genome mining revealed the presence of genes highly characteristic of plantaricin-related bacteriocin production (Table 2). This included genes for various plantaricins, specifically Plantaricin K, Plantaricin J, Plantaricin N, Plantaricin A, Plantaricin F, and Plantaricin E. Additionally, the analysis identified the gene for the plantaricin biosynthesis protein PlnO, genes for the response regulators PlnC and PlnD, the gene coding for the immunity protein PlnI (essential for self-protection), and genes coding for proteins associated with the bacteriocin export machinery (PlnG, PlnH). The high percentage identities and coverages across these various components of the plantaricin gene clusters strongly indicate a complete and functional plantaricin-like bacteriocin system.

Table 2. Gen matches detected in the assembled genome of *Lactiplantibacillus plantarum* CNTA 600 using BAGEL4 and ADP3 databases according to the % coverage and % identity of EFSA.

Database	Matched gene ID	Matched entry	%Coverage	%Identity
BAGEL4	Q88W84_LACPL	Bacteriocin immunity protein	100	99.068
BAGEL4	Q88W88_LACPL	Bacteriocin immunity protein	100	99.721
BAGEL4	173.2	Plantaricin_K	100	100
BAGEL4	172.2	Plantaricin_J	100	100
BAGEL4	P71462	Immunity protein PlnM	100	100
BAGEL4	174.2	Plantaricin_N	100	100
BAGEL4	P71464_LACPL	Plantaricin biosynthesis protein PlnO	100	99.749
BAGEL4	167.2	Plantaricin_A	100	100
BAGEL4	Q48829_LACPL	Response regulator PlnC, activator	100	100
BAGEL4	CAA64207.1	Response regulator PlnD	100	99.595
BAGEL4	P71468	Immunity protein PlnI	99	100
BAGEL4	171.2	Plantaricin_F	100	100

BAGEL4	170.2	Plantaricin_E	100	100
BAGEL4	Q88ZC2_LACPL	Bacteriocin ABC-transporter, ATP-binding and permease protein PlnG	100	100
BAGEL4	P71472_LACPL	Accessory factor for ABC-transporter PlnH	100	99.685
BAGEL4	Q88ZB7_LACPL	PlnY	100	100
BAGEL4	NP_786516.1	Bacteriocin immunity protein	100	99.153
ADP3	AP03290	BM1122 (natural AMPs; Bacteriocin, Gram-positive bacteria, prokaryotes, UCLL1)	100	80.22

3.1.2. SDS-PAGE and Zymography

The results of the SDS-PAGE assay performed with the CFS of *L. plantarum* CNTA 600 is shown in the Figure 2.a. Multiple protein bands were detected, including a strong band around 7 kDa.

When zymography was performed by confronting the gel band corresponding to *L. plantarum* CNTA 600 with the Gram-positive target bacteria *L. monocytogenes* CECT 7467, due to the antimicrobial activity observed by (Garin-Murguialday et al., 2024), a clear inhibition halo was observed (Figure 2.b). This halo was located at a position corresponding to a molecular weight between 4.6 and 10 kDa, strongly indicating the presence of a small protein with antimicrobial activity, i.e., a bacteriocin.

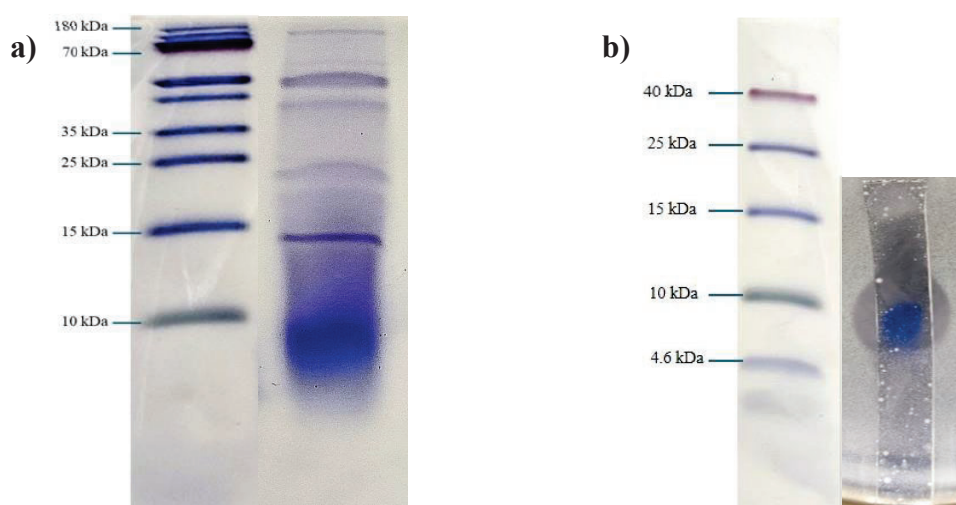


Figure 2. (a) Tricine/SDS-PAGE: (left) molecular marker PageRuler™ (10 to 180 kDa) and (right) protein band of *L. plantarum* CNTA 600. (b) Results from zymography assay: (left) molecular marker Protein Marker, Low Range (1.7 – 42 kDa). (b) inhibition halo showed by protein band of *L. plantarum* CNTA 600, where the gel as covered with viable cells of *L. monocytogenes* CECT 7467 (10^6 CFU/ mL) on TSA-YE agar incubated at 37°C for 24 hours.

This active band's size is consistent with various known *L. plantarum* bacteriocins, including the two-peptide complexes Plantaricin JK – composed of peptides PlnJ and PlnK – which is noted for its superior efficiency and selectivity, particularly in monovalent anion conduction (Abdulhussain Kareem & Razavi, 2020) and exhibits strong antimicrobial activity against Gram-positive bacteria. It demonstrates a broad spectrum of antimicrobial activity against pathogenic microorganisms by altering bacterial cell membrane permeability, specifically targeting monovalent ions like H⁺, which leads to bacterial cell death (Pal & Srivastava, 2014). This band could also correspond to Plantaricin EF, composed of the peptides PlnE and PlnF, which similarly shows significant activity against Gram-positive bacteria (D. Zhao et al., 2021). In summary, these zymography findings corresponded closely to the theoretical molecular weight of plantaricin JK (6.43 kDa) or plantaricin EF (7.25 kDa), although their precise identification requires further characterization.

3.1.3. Mass Spectrometry

Mass spectrometry analysis of the 6.43 kDa band of the CFS of *L. plantarum* CNTA 600 by mass spectrometry identified a unique peptide (UP), whose characteristics are summarized in Table 3. The gen *papC* is related to the chemical sections and pathways resulting in the formation of bacteriocins.

Table 3. Compounds identified by mass spectrometry from the gel band exhibiting antimicrobial activity.

MICROORGANISM NAME	Unique protein identification				
	Gen	UNIPROT ID	Length (Aa)	Molecular weight (kDa)	Function
<i>Lactiplantibacillus plantarum</i> CNTA 600	papC	A0A076G9U7	174,00	19.24	Chemical reactions and pathways resulting in the formation of a bacteriocin

* UNIPROT and NCBI were used to verify the molecular weights of the bacteriocins

3.2. Identification of the Spoilage Microorganisms

To identify the causes of the end of shelf life of the bites, samples were seeded on MRSA, DRBC and TSA-YE media throughout the 95-day shelf-life study.

No growth was detected on MRSA and DRBC media, which are selective for lactic acid bacteria and yeasts and moulds, respectively, indicating that the levels remained at zero, as in the initial product. Growth was observed exclusively on TSA-YE, in which two distinct colony morphologies were identified where the counts obtained were approximately 10 bacteria/g, indicating that although the heat treatment was insufficient, the microbial load of the final product was low. However, it was considered that this surviving population could be responsible for the sensory alterations reported by the company at the end of the product's shelf life.

From these colonies, molecular identification was performed through PCR sequencing of the 16S rRNA. The analysis identified the species that survived the pasteurization treatment of the bites as *Niallia circulans* and a member of the *Bacillus cereus* group, endospore-forming species that are capable to survive extrusion conditions (Cho & Chung, 2020; Florian Wild et al., 2014). These microorganisms have been described in the literature as spoilage agents for plant-based meat as described by (Barmettler et al., 2025) and (Tóth et al., 2021). For instance, *Bacillus thuringiensis* is widely used as a biocontrol agent in soybean plantations due to its insecticidal properties, which are effective against various insect pests (Kumar et al., 2021; Serrao et al., 2024; Snyder et al., 2024).

3.3. Antimicrobial Activity of the CFS against *Niallia circulans* and *Bacillus cereus*.

The antimicrobial activity of the CFS obtained by *L. plantarum* CNTA 600 was assessed using the modified time-kill curve method described by (Garin-Murguialday et al., 2024) enabling the determination of the MIC against the sporulated bacteria responsible of the product spoilage; Figure x for *N. circulans* and Figure x for group *B. cereus*.

3.3.1. Growth Inhibition and MIC of Spoilage Microorganisms

The time-kill curve demonstrated that the cell-free supernatant (CFS) of *L. plantarum* CNTA 600 exerted a concentration-dependent inhibitory effect on the growth of *N. circulans* (Figure 3) and group *B. cereus* (Figure 4). The control condition (absence of CFS, purple line), the bacterial population exhibited continuous growth, reaching the highest optical density (OD) close to 1.100 after 28 hours. In contrast, all tested CFS concentrations (0.15, 0.31, 0.625, 1.25, 2.5 y 5%) demonstrated varying degrees of growth inhibition. Notably, the highest concentrations (2.5% and 5%, pink and black lines, respectively) resulted in strong inhibition, with OD values remaining near baseline levels throughout the incubation period after an initial increase. This *in vitro* antimicrobial activity observed against *Bacillus* spp. was consistent with the findings reported by (S. Zhao et al., 2016) who determined the antimicrobial activity against *Bacillus* spp. of a *L. plantarum* strain isolated from the product Suan Tsai, or those described by (Akhtar & Nawaz, 2023; Nawaz, 2023), who demonstrated similar inhibitory effects of a CFS from a *L. plantarum* strain against *B. cereus*, *B. subtilis*, *S. aureus* and *E. coli* in their *in vitro* study.

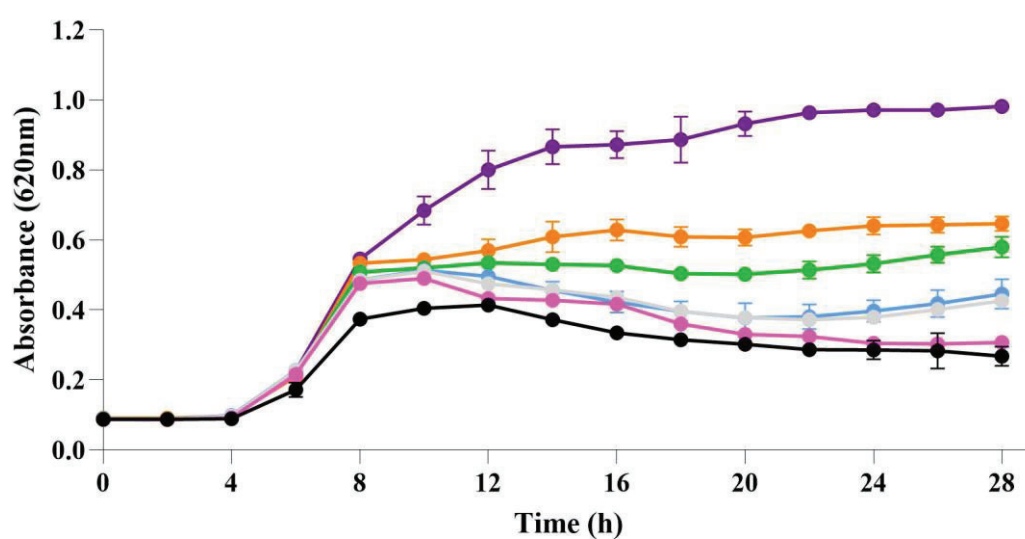


Figure 3. Evolution of the population of *N. circulans* strain isolated from the plant-based bites in TSB-YE incubated at 37°C for 28 hours in the absence of the CFS of *L. plantarum* CNTA 600 (purple colour), and in the presence of CFS of *L. plantarum* CNTA 600 at the final concentration of 0.15% (orange colour), 0.31% (green colour), 0.625% (blue colour), 1.25% (grey colour), 2.5% (pink colour) and 5% (black colour).

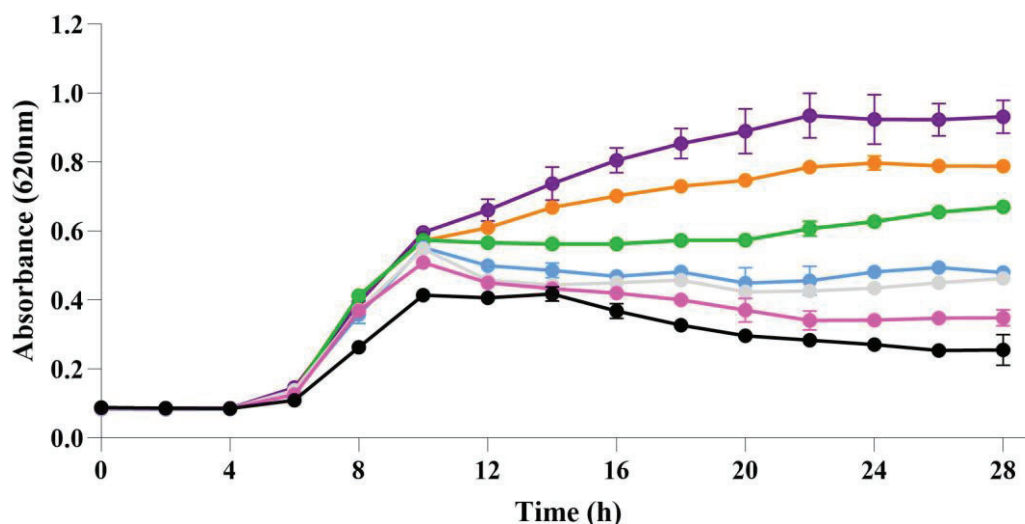


Figure 4. Evolution of the population of group *B. cereus* strain isolated from the plant-based bites in TSB-YE incubated at 37°C for 28 hours in the absence of the CFS of *L. plantarum* CNTA 600 (purple colour), and in the presence of CFS of *L. plantarum* CNTA 600 at the final concentration of 0.15% (orange colour), 0.31% (green colour), 0.625% (blue colour), 1.25% (grey colour), 2.5% (pink colour) and 5% (black colour).

3.4. *In Situ* Assessment of CFS Application in Plant-Based Chicken Bites

The results concerning sensory acceptance at time 0 are presented first, as the main objective of this analysis was to determine whether the addition of CFS negatively affected the initial sensory perception of the product, an essential factor for its potential consumer acceptance. This is followed by the microbiological analyses and, finally, the evaluation of physicochemical parameters.

3.4.1. Sensory Assessment of Plant-Based Bites by Consumer Panel

The cooked plant-based bites presented to the 11 panellists at CNTA corresponded to the time 0 days of the 4 batches produced and packaged by CNTA: i) control sample packaged in air atmosphere (20% vacuum) and subjected to the thermal pasteurization; ii) control sample packaged under MAP; iii) formulation containing the CFS of *L. plantarum* CNTA 600, packaged in air atmosphere (20% vacuum) and pasteurized; and iv) formulation with CFS of *L. plantarum* CNTA 600 packaged under MAP. However, based on the results of the physicochemical and microbiological analyses, this manuscript only reports the sensory perceptions and evaluations of the panellists for batches (i) and (iii).

The sensory panel was unable to detect any differences between the samples, rating the evaluated attributes as “typical” according to the tasting sheet. The addition of the CFS of *L. plantarum* CNTA 600 had no detectable impact on the sensory characteristics of the product.

The results showed that no significant differences were detected by the panellists between the 2 tested batches: the control and the one formulated with the CFS of *L. plantarum* CNTA 600. This suggested that the use of the CFS did not alter the sensory perception of the product, maintaining characteristics comparable to those of the control or original formulation.

3.4.2. Effects of CFS Treatment on Microbial Proliferation of Plant-Based Bites

Monitoring the natural microbial development in the plant-based bites was essential for determining the most effective preservation strategy to extend the bites shelf life. Therefore, mesophilic aerobic endospore-forming bacteria, responsible for spoilage, were monitored over a 95 days of storage period. Additionally, to ensure that the concentration of moulds and yeasts, and LAB remained below detection limits, these microbial groups were also monitored with fewer sampling points (data not shown).

At day 0, immediately after the production of the plant-based bites, significant differences were observed in the counts of mesophilic aerobic endospore-forming bacteria among the different packaging and treatment conditions (Table 5). Samples packed in air and subjected to heat treated - both the control and the CFS of *L. plantarum* CNTA 600-treated batches - showed lower bacterial counts (1.10 ± 0.17 and 1.15 ± 0.21 log CFU/g, respectively) compared to those packed under MAP conditions without heat treatment, which exhibited higher counts in both control (3.32 ± 0.35 log CFU/g) and the CFS-treated samples (3.43 ± 0.23 log CFU/g). These findings highlighted the effect of

the thermal treatment on vegetative cell inactivation, later to become endospores (Klijn et al., 2025).

After the first 30 days at 4°C, corresponding to the product's intended shelf-life, the non-heat-treated MAP samples exceeded the regulatory limit of 10^5 CFU/g for mesophilic aerobes in ready-to-eat products (Comission, 2005), reaching 8.05 ± 0.15 log CFU/g in the control and 7.54 ± 0.20 log CFU/g in the batch treated with the CFS of *L. plantarum* CNTA 600 (Table 5). These values were significantly higher than those observed in the heat-treated samples, which remained at 2 log CFU/g.

Consequently, monitoring of the MAP samples was discontinued beyond this point, and focus shifted to the air-packed and heat-treated batch. These results underscored the necessity of applying a thermal treatment to the packed product to ensure microbial stability during the storage.

Table 5. Aerobic mesophilic counts at day 0 and day 30 during the shelf-life study of plant-based bites.

Time (days)	Mesophilic aerobic counts (CFU/ g)			
	control air + heat treatment	control + MAP	CFS of <i>L. plantarum</i> CNTA 600 + air + heat treatment	CFS of <i>L. plantarum</i> CNTA 600 + MAP
0	$1.10^e \pm 0.17$	$3.32^c \pm 0.35$	$1.15^e \pm 0.21$	$3.43^c \pm 0.23$
30	$2.00^d \pm 0.00$	$8.05^a \pm 0.15$	$2.00^d \pm 0.00$	$7.54^b \pm 0.20$

Therefore, the shelf-life study of the plant-based bites was limited to monitoring the evolution of mesophilic aerobic bacteria in 2 batches: the control + air-packed + heat-treatment, and the CFS of *L. plantarum* CNTA 600 + air-packed + heat-treated.

These results are presented in Figure 6, which shows that during the first 40 days of refrigerated storage (4°C for 30 days followed by 8°C for 65 days), the mesophilic aerobic population in both batches evolved similarly, with bacterial counts increasing to approximately 10^2 CFU/ g. However, beyond this time the microbial behaviour diverged, in which the temperature was of 8°C.

In the control packaging, where the only preservation barriers were 20% vacuum and thermal treatment, the mesophilic aerobic population increased by 7 log cycles CFU/ g over the final 55 days of storage. The most pronounced microbial growth occurred between days 50 and 65, during which the product transitioned from being within acceptable microbiological limits defined by the EU Regulation (EC) No. 2073/2005 (Comission, 2005) to exceeding them and becoming a non-consumable product. This grow in the mesophilic population continued until reaching a concentration of 9.53 ± 0.13 log cycles CFU/ g after the 95 days of the study at refrigerated storage.

In contrast, the microbial evolution of the *plant-based* bites formulated with the CFS of *L. plantarum* CNTA 600, vacuum-packed (20%) and heat-treated, was more moderate. As with the control samples, the most notable increase in mesophilic aerobic concentration as between days 50 and 65, but in this case reaching 3.91 ± 1.08 log cycles CFU/ g. By the final day of the study, day 95, the microbial load reached 5.08 ± 0.32 log cycles CFU/ g, in the limit established by the EU Regulation (EC) No. 2073/2005 (Comission, 2005).

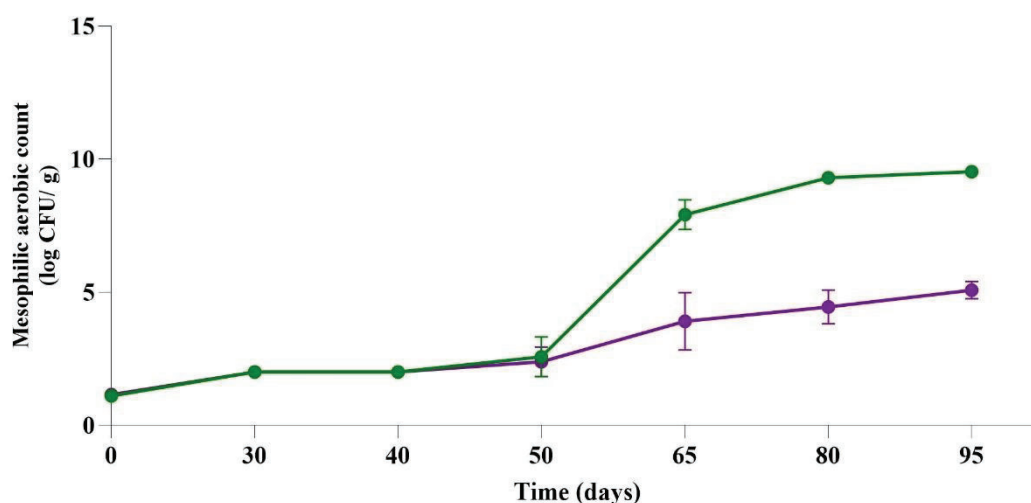


Figure 6. Evolution of the spoilage microbial population (spore-forming bacteria from the *B. cereus* group and *N. circulans*) in the control plant-based bites (purple colour) and the bites treated with 5% v/w liquid-format CFS from *L. plantarum* CNTA 600 (green colour), stored for 95 days (4°C for 30 days + 8°C for 65 days).

In conclusion, the selected preservation strategy to extend the shelf life of the plant-based bites consisted of formulating the soy mixture with 5% v/w of the CFS of *L. plantarum* CNTA 600, packaging under air atmosphere with 20% vacuum, and applying a thermal pasteurization treatment.

3.4.3. Impact of CFS Treatment on the Physicochemical Properties of Plant-Based Bites

The monitoring of pH and headspace gas composition was carried out exclusively on the batches packaged in air, control and CFS-treated ones, based on the findings of the microbiological analysis. This selective approach aimed to assess the evolution of the physicochemical parameters of this plant-based bites batches.

3.4.3.1. pH evolution of the plant-based bites

The pH measurements taken at time 0 days, immediately after the production of the plant-based bites, indicated that the addition of the CFS from *L. plantarum* CNTA 600 did not result in a significant acidification of the samples although the CFS itself having a pH of 4.44 (Garin-Murguialday et al., 2024). The control samples (control air + heat treatment) showed a pH of 7.06 ± 0.06 , while the CFS-treated bites a pH of 6.99 ± 0.03 . The pH evolution, shown in Table 4, was monitored throughout 95-day refrigerated storage (4°C/ 30 days + 8°C/ 65 days). Although the results remained relatively stable over time, statistical analysis revealed some significant differences between specific time points within each treatment group. However, no consistent trend of acidification was observed, indicating good stability under the tested conditions. The overall pH profile of the CFS-treated samples (CFS of *L. plantarum* CNTA 600 + air + heat treatment) remained comparable to that of the control group, both maintaining values close to neutrality (Bonaldo et al., 2023; Tóth et al., 2021).

Table 4. pH evolution of the control batch and the CFS-treated batch plant-based bites during 95 days at refrigerated conditions.

Time (days)	Sample batch	
	Control plant-based bites	CFS-treated plant-based bites
0	7.06 ^a ± 0.06	6.99 ^{a,b,c,d} ± 0.03
30	7.07 ^a ± 0.03	7.01 ^{a,b} ± 0.01
40	6.99 ^{a,b,c,d} ± 0.02	6.90 ^{b,c} ± 0.05
50	6.84 ^e ± 0.13	6.86 ^{d,e} ± 0.03
65	7.00 ^{a,b,c} ± 0.05	6.87 ^{c,e} ± 0.01
80	7.00 ^{a,b,c} ± 0.02	6.88 ^{b,e} ± 0.02
95	6.81 ^e ± 0.04	6.86 ^{d,e} ± 0.02

3.4.3.2. Analysis of headspace gas composition

As shown in the Figure 5, significant changes in the gas composition were observed during refrigerated storage (4°C/ 30 days + 8°C/ 65 days) in the control batch. For this samples the oxygen concentration ([O₂]) remained relatively stable and comparable up to day 50. However, at this point, a noticeable decline in [O₂] was detected, accompanied by a corresponding increase in carbon dioxide concentration ([CO₂], suggesting of ongoing metabolic activity. The growth of mesophilic aerobic microorganisms is directly related to the presence of oxygen in the environment, as these organisms require oxygen to carry out their metabolisms and reproduce (Ojha et al., 2024; Rajanikar et al., 2021). In contrast, the batch formulated with the CFS of *L. plantarum* CNTA 600 exhibited no significant differences in either [O₂] or [CO₂] throughout the 95 days of the study period. This stability suggested a lack or low microbial activity under the tested storage conditions, what indicated the inhibitory properties of the CFS *L. plantarum* CNTA 600 in plant-based bites.

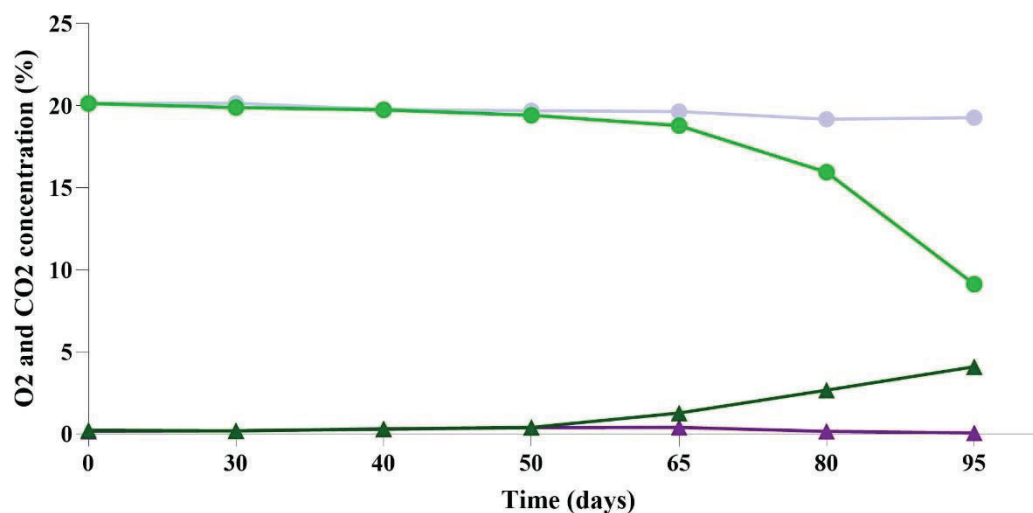


Figure 5. Changes in the headspace gas composition during the storage of plant-based bites packed in PP/EVOH/PP plastic trays and sealed with PP/EVOH/PP film, containing 20% vacuum, stored for 30 days at 4°C followed by 65 days at 8°C: control bites [O₂] (light green) and [CO₂] (dark green); and bites treated with 5% v/w liquid SLC from *L. plantarum* CNA 600 [O₂] (light purple) and [CO₂] (dark purple).

4. CONCLUSIONS

The incorporation of the liquid-format CFS from *L. plantarum* CNA 600 into the formulation of plant-based bites, combined with subsequent packaging under 20% vacuum in PP/EVOH/PP plastic trays sealed with the same multilayer film, effectively inhibited the growth of the two spore-forming bacteria associated with the product spoilage *N. circulans* and group *B. cereus* after 95 days of refrigerated storage in comparison to the control batch. The addition of this CFS to the soy protein matrix extended the shelf life of the plant-based bites from 30 to 95 days. This shelf-life extension was primarily attributed to the production of lactic and acetic acids, as well as the synthesis of the bacteriocin plantaricin. These results were confirmed by *in vitro* assays, bioinformatic analyses, and molecular characterization conducted throughout the shelf-life study of the plant-based bites. Notably, the strain used in this study complies with the safety criteria established by the European Commission (EC): it belongs to the QPS list, exhibits no transferable antibiotic resistance, and has been characterized as non-pathogenic in accordance with the described by Garin-Murguialday et al. (2024).

Therefore, the SLC of the strain *L. plantarum* CNTA 600 stands out as a sustainable and natural alternative to replace E-number preservatives in plant-based meat analogues, owing to its proven antimicrobial activity and clean-label potential.

Declaration of interests

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Acknowledgements

The University, Innovation and Digital Transformation Department, R&D&I 638 Service of the Government of Navarra (Spain) provided N. Garin-Murguialday with a grant to carry out this research.

Funding

This research was funded by Gobierno de Navarra, funding allocation G20001-642 G2100-4701- 467300: Contratación personal investigador y tecnológico, doctorados 643 industrials—project BacBiop (0011-1408-2022-000003). This research was also funded by MCIN/AEI/10.13039/501100011033, Grant PID2021-123404NB-I00, by “ERDF A 645 way of making Europe” and by the Government of Aragón (grant Grupo AESA).

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The raw sequencing reads were deposited in the European Nucleotide Archive (accession number of the project: PRJEB74905, Biosample SAMEA118340401). The rest of the raw data is available at the provided link:

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Manuscrito VII

Cultivos protectores potencialidades y el reto del etiquetado

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Cultivos protectores: potencialidades y el reto del etiquetado

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Los cultivos protectores son microorganismos que se pueden emplear en alimentos fermentados y no fermentados, para inhibir el crecimiento de microorganismos indeseables, patógenos y alterantes y garantizar la inocuidad y/o prolongar la vida útil del producto. Su uso presenta gran potencial dentro de la industria alimentaria, aunque un reto pendiente es la clarificación del etiquetado que varía según se consideren parte del proceso o un ingrediente *active* (aditivo)

Uno de los retos a solventar en el uso de cultivos protectores como estrategia *clean label* es el etiquetado. La regulación alimentaria recoge distintas casuísticas dependiendo del tipo de alimento al que se añada el cultivo protector y el formato en el que este es incorporado.

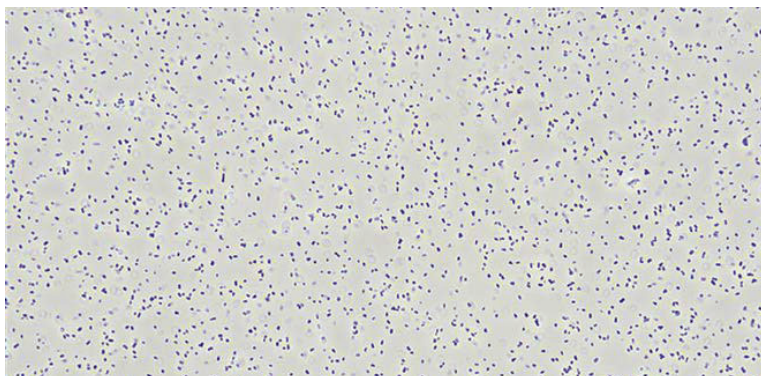
En alimentos fermentados, los cultivos protectores no suelen incluirse en la lista de ingredientes al considerarse

parte del proceso tradicional de fermentación. En el marco de la legislación europea, y concretamente según el Reglamento (UE) nº 1169/2011, estos cultivos pueden considerarse coadyuvantes tecnológicos, siempre que se utilicen con la función tecnológica propia del *starter* (cultivo iniciador) propuesto para llevar a cabo la actividad fermentativa y no ejerzan un efecto tecnológico adicional en el alimento final.

Aunque estos microorganismos, tras la fermentación, permanezcan en el

producto terminado —como ocurre en embutidos fermentados, quesos o yogures—, su presencia no implica que deban declararse en el etiquetado siempre que no se les atribuya una función activa en el producto final ni un efecto nutricional o de salud. Esta consideración es habitual cuando los cultivos protectores se emplean junto con el cultivo iniciador y forman parte del proceso tradicional de fermentación.

En alimentos no fermentados la situación se complica. La adición de

FIGURA 1. *P. ACIDILACTICI* CNTA 1059 AL MICROSCOPIO

estos cultivos protectores busca incrementar la vida útil de productos mínimamente procesados distribuidos en refrigeración. La adición de cultivos “vivos” en estos alimentos supone:

1) que estos cultivos continúan metabólicamente activos durante el almacenamiento en refrigeración y provocan la alteración del producto.

2) que los recuentos de mesófilos aerobios (indicador de calidad microbiológico habitual en las empresas) en el producto final supere los límites recomendados por la regulación puesto que el cultivo protector se contabiliza en ese recuento.

Por estos dos motivos, es cada vez más habitual que las empresas ingredientistas distribuyan como cultivos protectores cultivos bacterianos inactivos (potenciales postbióticos) y/o el Sobrenadante Libre de Células (SLC) de los cultivos una vez eliminada la biomasa bacteriana.

Estas alternativas a los cultivos vivos (que solventan los dos inconvenientes descritos anteriormente) plantean la siguiente cuestión en el etiquetado: ¿debería declararse como ingrediente con efecto antimicrobiano o podría etiquetarse como “aroma” si colateralmente al efecto conservante supone un cambio significativo y deseable en el aroma del producto?

Si se opta por añadir el SLC o el cultivo inactivo, podrían contemplarse 4 opciones de etiquetado:

1. Si el efecto principal es la inhibición de microorganismos y permanece durante la vida útil, se encuadra en la definición de “aditivo alimentario/ con-

servador”, según el Reglamento (CE) nº 1333/2008. Para etiquetarse como tal, el compuesto activo debe estar aprobado y listado en el anexo II del reglamento con un número E (por ejemplo, la nisina, E 234). Si no lo está, se deberán llevar a cabo los pasos establecidos para que el SLC se reconozca “nuevo aditivo” al que se le asignaría un número E y un nombre, ej. “cultivo protector (*Lactiplantibacillus plantarum*)”.

2. Etiquetar como aroma alimentario, en caso de demostrar que el ingrediente añadido (SLC o cultivo bacteriano inactivo) ofrece un cambio sensorial perceptible en el producto final y que esta es su actividad principal. El etiquetado sería “aroma X” de acuerdo a como se indica en el Reglamento (CE) nº 1334/2008.

3. El Reglamento (UE) 1121/2010 menciona explícitamente “fermentos de microorganismos productores de ácido láctico y de aroma” como subcategoría dentro de ciertos alimentos. Esto indica que dicho término está reconocido a

nivel de la UE y puede emplearse en el etiquetado para ingredientes microbianos específicos, pero, en el caso del SLC, no se ajustaría a esta categoría porque: a) no contiene células microbianas activas; b) su función principal no es la fermentativa.

4. En caso de que el SLC o el cultivo inactivo no se considere un aditivo aislado sino un “caldo postbiótico” con múltiples componentes, podría argumentarse que se trata de un ingrediente funcional de origen microbiano. Sin embargo, en ausencia de un historial de consumo significativo antes de mayo de 1997, el SLC podría clasificarse como *novel food* conforme al Reglamento (UE) nº 2015/2283, requiriendo evaluación por parte de EFSA y autorización por parte de la Comisión.

¿Dónde se utilizan cultivos protectores en la industria alimentaria?

En la actualidad, diversos sectores alimentarios (principalmente de alimentos fermentados) aplican microorganismos para garantizar la seguridad y la calidad del producto, así como alargar su vida útil. Algunos ejemplos en este sentido se pueden ver en la industria cárnica, donde diferentes empresas han desarrollado productos como embutidos en los que se utilizan cultivos protectores para inhibir patógenos. Algunos fabricantes han optado por incluir en menciones como “cultivo iniciador” o “cultivo protector” para evidenciar la seguridad microbiológica del producto, pero no existe una definición específica ni autorización en la legislación alimentaria europea que habilite el uso de la denominación

FIGURA 2. PRODUCTOS FERMENTADOS EN LOS QUE SE UTILIZAN CULTIVOS PROTECTORES PARA GARANTIZAR SEGURIDAD SANITARIA E INCREMENTAR VIDA ÚTIL



“cultivo protector” en el etiquetado.

El término se usa en la práctica técnica —por ejemplo, para bacterias lácticas que inhiben patógenos— pero no está integrado en los reglamentos de ingredientes o aditivos. Según Reglamento (UE) 1169/2011, los ingredientes con actividad tecnológica o funcional durante la fabricación deben listarse usando una denominación generalmente comprendida por lo que la opción legal sería incluir en la etiqueta: *Starter*: cultivos lácticos (*Lactobacillus* sp., *Pediococcus* sp.) o Cultivo iniciador (*Lactiplantibacillus plantarum*, etc.) y opcionalmente una nota aclaratoria en ingredientes técnicos: “incluye cultivos protectores para garantizar seguridad microbiológica”. Esto añade transparencia sin violar denominaciones oficiales.

En el sector de alimentos *ready to eat* (RTE), algunas empresas añaden cultivos iniciadores de bacterias lácticas —incluyendo cepas con estatus QPS reconocidas por EFSA— o preparados microbianos inactivados con función tecnológica, para prolongar la vida útil y reforzar la seguridad microbiológica mediante mecanismos fermentativos o conservantes naturales. En algunos casos, el etiquetado indica la ausencia de conservantes químicos en el sentido de que no se han añadido preservantes sintéticos autorizados (Reglamento 1333/2008), gracias al uso de estos preparados. Estas declaraciones pueden calificarse como declaraciones tecnológicas, siempre que se cumplan los criterios del Reglamento 1169/2011 y no impliquen funciones de salud o nutrición sujetas a autorización bajo el Reglamento 1924/2006.

El caso de un producto no fermentado: Gazpacho

En el marco del proyecto BACBIOP, financiado por Gobierno de Navarra y según el cual se está desarrollando una tesis doctoral, CNTA ha llevado a cabo las pruebas para cuantificar las posibi-

FIGURA 3. LLENADO DE LOS ANACLINES CON EL GAZPACHO.



lidades del uso del SLC de la bacteria láctica *Levilactobacillus brevis* CNTA 1374 para inhibir el crecimiento de las bacterias, lácticas también, responsables del fin de vida útil del gazpacho fresco (sin tratamiento térmico).

Al no ser un alimento fermentado, queda descartado su etiquetado como “*starter* de fermentación” y según lo anteriormente descrito, las posibilidades serían:

a) si se cultiva la bacteria y de su Sobrenadante Libre de Células (SLC), se purifican e identifican los compuestos activos (incluidos en la lista positiva de aditivos conforme al Reglamento (CE) n° 1333/2008) y se añade directamente, se debe etiquetar como aditivo, con su correspondiente número E,

b) si se cultiva la bacteria y, tras centrifugación, se elimina la bacteria (biomasa microbiana) y se utiliza el SLC como ingrediente para ejercer la actividad antimicrobiana, habría 3 vías:

Vía 1: realizar las gestiones para que ese SLC sea reconocido como aditivo alimentario seguro y una vez conseguido incluir el número E asignado (o el nombre autorizado) en la etiqueta del producto.

Vía 2: optar por el etiquetado como “aromas” basándose en el Reglamento

(CE) n° 1334/2008. No obstante, si el objetivo principal es la seguridad microbiológica y no una modificación sensorial, esta clasificación es arriesgada y podría ser cuestionada por las autoridades.

Vía 3: el SLC podría clasificarse como novel food conforme al Reglamento (UE) n° 2015/2283, requiriendo evaluación por parte de EFSA y autorización por parte de la Comisión.

Conclusión

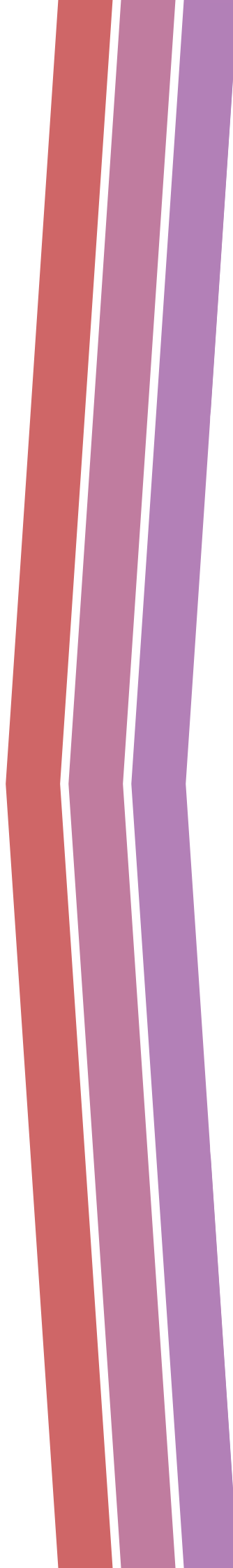
El uso de microorganismos y sus derivados como estrategia bioconservante representa una oportunidad real para desarrollar alimentos más seguros y con una vida útil prolongada, alineándose con la demanda de productos *clean label*. En particular, el empleo del sobrenadante libre de células (SLC) de bacterias

lácticas se perfila como una alternativa funcional a los cultivos vivos, especialmente en alimentos no fermentados. Sin embargo, su incorporación como ingrediente plantea retos regulatorios relevantes que dependen de su función tecnológica y del impacto sensorial que genera en el alimento.

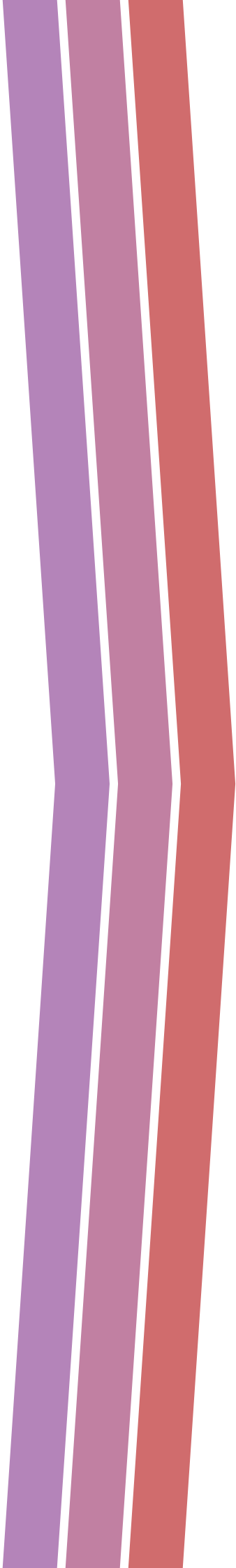
En este contexto, es crucial diseñar una estrategia de desarrollo que tenga en cuenta desde el inicio las implicaciones regulatorias. A futuro, si se reconoce el concepto de “postbiótico” en la legislación alimentaria de la UE, y el SLC procede de una cepa con estatus QPS, podría abrirse una vía adicional de uso como ingrediente funcional postbiótico. No obstante, mientras tanto, la clasificación legal más adecuada dependerá de pruebas funcionales, sensoriales y de seguridad que justifiquen su uso en cualquiera de las categorías actualmente reconocidas. □

***El Gobierno de Navarra ha financiado la contratación en CNTA de la doctoranda Nerea Garín en el marco de la convocatoria de ayudas ‘Doctorados Industriales 2022’.**

El trabajo que se describe en este artículo se está realizando gracias a la ayuda para la realización de Tesis Doctorales del Gobierno de Navarra.



CONCLUSIONES



Conclusiones

Considerando los objetivos definidos en esta tesis doctoral, a continuación, se exponen las conclusiones derivadas del análisis de los resultados obtenidos:

1. Un *screening* inicial de todas las cepas de la colección de CNTA permitió identificar a 253 cepas bacterianas con estatus QPS y no sujetas al Protocolo Nagoya con potencial como cultivos protectores. De estas, se han seleccionado y caracterizado los sobrenadantes libres de células (SLC) de 4 cepas por su espectro de actividad antimicrobiana, tanto frente a bacterias responsables de toxiinfecciones alimentarias como frente a microorganismos alterantes de alimentos mínimamente procesados. De este modo, la adición de cultivos protectores de *Pediococcus acidilactici* CNTA 1059, *Latiplantibacillus plantarum* CNTA 600, *Levilactobacillus brevis* CNTA 1374 y *Bacillus subtilis* CNTA 517 constituye una estrategia de conservación *clean label*, es decir, con el objetivo de reducir (o incluso eliminar) el uso de conservantes con número E-.
2. La actividad antimicrobiana del SLC de la cepa *B. subtilis* CNTA 517 se atribuye a la presencia de bacteriocinas: subtilosina A (3,40 kDa), bacilicina (0,27 kDa), fengicina (1,46 – 1,50 kDa), bacillibactina (0,88 kDa), surfactina (1,04 kDa), pelgipeptina (1,07 kDa) y de ácidos orgánicos como el ácido láctico ($2,70 \pm 0,17$ g/L) y ácido acético ($< 0,25$ g/L).
3. La actividad antimicrobiana de la cepa *L. plantarum* CNTA 600 se atribuye a la presencia de las posibles bacteriocinas plantaricina EF y JK, de tamaño molecular 7,25 y 6,43 kDa, respectivamente, y de ácido láctico ($7,39 \pm 0,13$ g/L) y ácido acético ($3,64 \pm 0,00$ g/L).

4. La actividad antimicrobiana del SLC de la cepa *P. acidilactici* CNTA 1059 se atribuye a la presencia de la bacteriocina pediocina PA-1 de tamaño molecular 4,6 kDa, y a la presencia de ácido láctico ($8,20 \pm 0,19$ g/L) y ácido acético ($3,58 \pm 0,07$ g/L).
5. La actividad antimicrobiana del SLC de la cepa *L. brevis* CNTA 1374 se atribuye a la presencia de las bacteriocinas categorizadas como RiPP-like y lantipéptido clase II, de tamaño molecular < 10 kDa en ambos casos, y a la presencia de ácido láctico ($2,80 \pm 0,02$ g/L) y ácido acético ($3,78 \pm 0,07$ g/L).
6. Se ha definido una estrategia *clean label* para el desarrollo y conservación de sticks de piña de IV gama de la empresa Florette Ibérica basada en la inmersión de las piezas de piña en el SLC de la cepa *P. acidilactici* CNTA 1059 con el objetivo de alcanzar una concentración del SLC en la pieza de fruta del 5% v/p y el posterior envasado en atmósfera MAP pasiva con plástico microperforado. Con esta estrategia la vida útil de la piña envasada se extendió de los 8 días actuales a, al menos, 12 días.
7. Se ha definido una estrategia de conservación *clean label* para el desarrollo y conservación de los bocados *plant-based* de la empresa Sanygran basada en la incorporación del SLC de *L. plantarum* CNTA 600 al 5% v/p en la formulación, combinada con un envasado al vacío del 20% y un tratamiento térmico de pasteurización. Con esta estrategia la vida útil de los bocados *plant-based* se extendió de los actuales 30 días hasta los 95 días.
8. En las condiciones ensayadas, la estrategia *clean label* basada en la adición de un 5% v/v del SLC de *Levilactobacillus brevis* CNTA 1374 a gazpacho fresco de la empresa AMC no consiguió incrementar la vida útil más allá de los 60 días que se logra con el tratamiento térmico suave sin la adición de conservantes.

9. El etiquetado de los alimentos en los que se incorpora el SLC de los cultivos protectores como estrategia *clean label* depende de que i) el SLC esté totalmente caracterizado y sus partes estén reconocidas en la lista positiva de aditivos, en cuyo caso se deberá etiquetar con el correspondiente número E; ii) el SLC aporte un cambio aromático deseable y perceptible en el producto final, en cuyo caso se podría etiquetar como aroma; iii) el SLC se considere un ingrediente más de la formulación del alimento, en cuyo caso se podría clasificar como *Novel Food*, lo que requeriría una evaluación de seguridad por parte de EFSA y la correspondiente autorización por parte de la Comisión.
10. Esta tesis doctoral ha contribuido a establecer nuevas metodologías de trabajo internas dentro de la línea de Nuevas Estrategias y Tecnologías de Conservación (NETC) de CNTA, lo que ha permitido ampliar la oferta de servicios del centro en el ámbito de la conservación de alimentos, fortaleciendo, de este modo, el apoyo a la industria alimentaria.

